

Binding geometry, stoichiometry, and thermodynamics of cyclomalto-oligosaccharide (cyclodextrin) inclusion complex formation with chlorogenic acid, the major substrate of apple polyphenol oxidase

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ABSTRACT

The inclusion complexes of cyclomaltohexaose (α -CD), cyclomaltoheptaose (β -CD), cyclomaltooctaose (γ -CD), and polymerized β -CD (β -CD_n) with chlorogenic acid (CA), the major substrate of apple fruit polyphenol oxidase (PPO), were studied with regard to pH, ionic strength, and temperature in model buffer systems and apple juice. The thermodynamics of CD·CA inclusion complex formation, which were studied in solution using UV spectrophotometry, displayed enthalpy–entropy compensation typical of processes driven by solvation phenomena. We also found that the apparent association constants (K) of the CD·CA equilibrium were relatively insensitive to pH for β -CD, compared to α - and γ -CDs, but were subject to substantial enhancement at low ionic strengths. The β -CD·CA inclusion complex was also characterized for binding geometry and stoichiometry at 9.4 T and 25°C in 0.05 M Na phosphate buffer by ¹H NMR spectroscopy. A 1:1 stoichiometric ratio for the complex was found using the method of continuous variations. ¹H Spin–lattice relaxation and chemical-shift data indicate that the phenolic ring of CA docks within the cavity of β -CD. The K s for β -, α -, and γ -CD determined in apple juice, which contains a mixture of PPO substrates, were found to correlate with PPO activity-related data. Apple juice, treated with β -CD_n, did not brown until CA was added back. These latter findings strongly argue that the mechanism for inhibition of juice browning with cyclodextrins was mainly due to the binding of PPO substrates and not some other means such as enzyme inactivation via sequestration of Cu²⁺ by CDs.

INTRODUCTION

The control of enzymatic browning in fresh fruits and vegetable products presents a problem for the food processing industry since the utilization of sulfites

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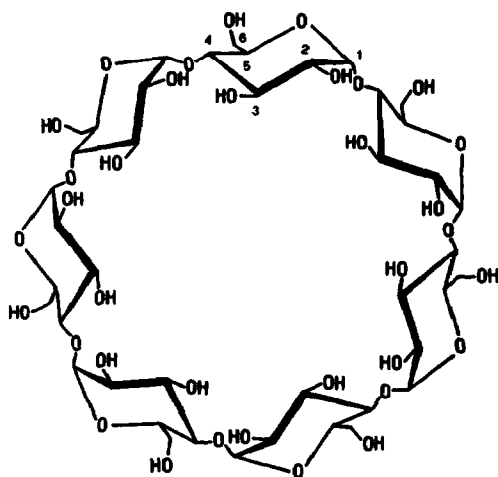
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has been restricted by the U.S. Food and Drug Administration (FDA)¹. Cyclomalto-oligosaccharides (cyclodextrins or CDs; dp 7, 1), which form inclusion complexes with manifold guest molecules², have been shown to inhibit enzymatic browning in fresh fruit and vegetable juices^{1,3}. In these studies the authors proposed that CDs might sequester the substrates of apple polyphenol oxidase (PPO) via the formation of inclusion species, thereby preventing their enzymatic oxidation and subsequent condensation to form undesirable pigments. However, it is also possible that the mechanism of browning inhibition by CD treatment relates to the chelation of Cu^{2+} , an essential component for PPO activity. Although it is known that CDs form discrete inclusion complexes with some polyphenols⁴, cinnamic acid⁵, and other guest molecules^{2,6}, the formation of inclusion complexes between CDs and chlorogenic acid^{7,8} (CA, **2**), an important substrate for PPO in apples, has not been fully documented. Basic knowledge gained in this area could be useful in the prediction and identification of natural anti-browning oligo- and poly-saccharides which are likely to be FDA approved and commercially acceptable. To this end, our present work is mainly focused on the thermodynamic characterization of the inclusion complexes of α -CD (1, dp 6), β -CD (1), epichlorohydrin-polymerized β -CD⁹ (β -CD_n), and γ -CD (1, dp 8) with **2** as a function of temperature, pH, and ionic strength in model buffer systems and apple juice. The β -CD · CA complex was also characterized for binding geometry and stoichiometry using ¹H NMR spectroscopy.

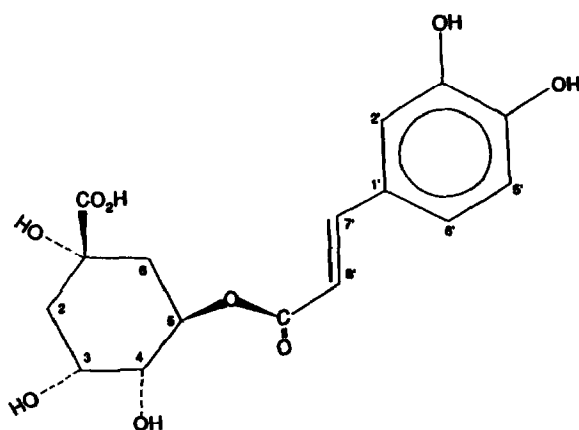
EXPERIMENTAL

General.—Samples of β -CD, β -CD_n, and γ -CD were donated by the American Maize-Products Company (1100 Indianapolis Blvd., Hammond, IN 46320-1094, USA). α -CD, CA {1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-[3,4-dihydroxycinnamic acid]}, (–)-epicatechin {*cis*-2-[3,4-dihydroxyphenyl]-3,4-dihydro-2*H*-1-benzopyran-3,5,7-triol}, L-tyrosine, and 2,6-dihydroxybenzoic acid were purchased from Sigma Chemical Company. 3,4-Dihydroxybenzoic acid and rutin were from Aldrich Chemical Company. DL-Tryptophan and 2,5-dihydroxybenzoic acid were obtained from E. Merck and Eastman Laboratory Chemicals, respectively.

¹H NMR experiments.—All studies were performed on a Jeol NMR spectrometer operated at 400 MHz (B_0 9.4 T) in 99.9 atom-% D₂O (MSD Isotopes; 0.05 M Na Phosphate buffer, pD = pH + 0.4 = 6.9). Typical spectrometer conditions were: 32 K data points; 3.5 kHz spectral width; 6 μ s pulse width (90° pulse = 11 μ s). Chemical shifts were assigned relative to water (4.75 ppm). The ¹H NMR resonance assignments of **2** were made based upon a homonuclear COSY experiment and qualitatively agree with those made by Merfort¹⁰ for the caffeoyl derivative of **2**. For the measurement of the apparent association constant, K (25°C), **2** was maintained at a concentration of 1.25 mM and β -CD concentrations were varied between ca. 0 and 11 mM. ¹H Inversion recovery (T_1) peak intensity data were fitted to the usual exponential equation utilizing a modified Gauss–Newton¹¹



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algorithm on a Microsoft Excel spreadsheet created in this laboratory; 10 τ values, the time between the 180° and 90° pulses, of 25 ms to 4 s were used. For the continuous variations studies (Job's plots)^{12,13}, the total concentration of 2 + β -CD was 10 mM, and individual component concentrations were altered to produce mole fractions (χ) such that $\chi_{\beta\text{-CD}}$ or χ_{CA} were 0 to 1 in increments of 0.1.

UV experiments.—Most of the UV studies reported herein were performed on a Perkin–Elmer 552 dual-beam spectrophotometer. Thermodynamic experiments utilized a Neslab Endocal refrigerated circulating water bath to maintain temperatures at $\pm 2^\circ\text{C}$. Samples were maintained at a particular temperature for at least 1 h to ensure the attainment of thermal equilibrium. CD Complex kinetics are

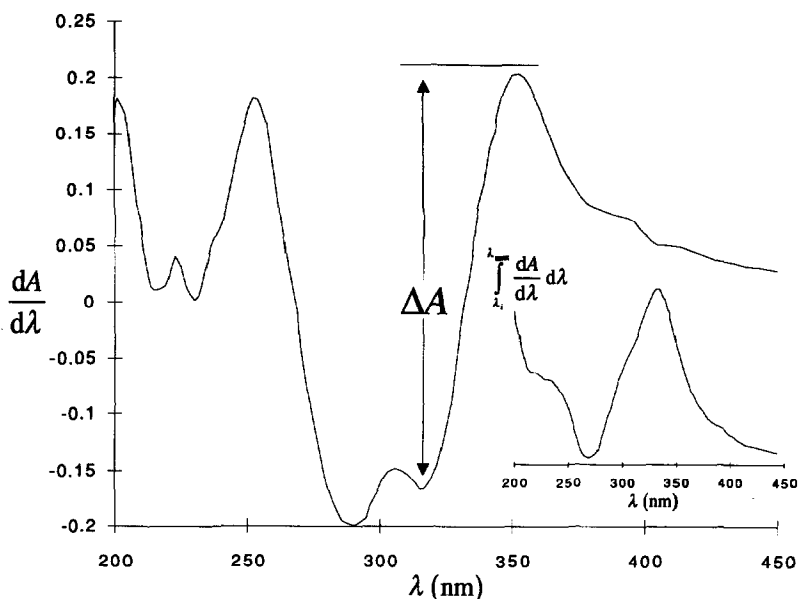


Fig. 1. UV difference spectrum of the α -CD·CA inclusion complex ($[\alpha\text{-CD}]_0 = 1.2 \times 10^{-2}$ M; $[\text{CA}]_0 = 8 \times 10^{-5}$ M; 0.1 M Na phosphate buffer); inset, integration of difference spectrum.

typically fast (2.8 to $10^8 \text{ M}^{-1} \text{ s}^{-1}$)¹⁴, and a chemical equilibrium is approached quickly ($20 \mu\text{s}$ to 8 min). For measurement of K_s , the concentrations of guest species were fixed at 8×10^{-5} M; CD concentrations were typically $0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 10$, and 12 mM . Either 0.1 M Na phosphate or acetate buffers were used ($\text{pH } 6.5$ or 3.6 , respectively). For soluble CD experiments, data were recorded as difference spectra; the reference cuvette contained the appropriate buffer + guest while the sample cell contained all these components + CD. Working with **2**, we found that, unlike some guest^{5,14} molecules, peak amplitude did not change much as a function of binding. However, **2** was similar to other guest species^{5,14} in the presence of CD since significant, $3\text{--}6 \text{ nm}$ ($\Delta\lambda_{\text{max}}$), bathochromic shifts in the wavelength of maximum absorbance were detected. Thus, the difference spectrum ($[\text{2} + \text{CD}] - [\text{2}]$) displayed a typical first-derivative appearance, as illustrated in Fig. 1 ($\text{pH } 6.5$, $12 \text{ mM } \alpha\text{-CD}$), because the subtraction of any two absorbance manifolds, half of which are offset by $\Delta\lambda_{\text{max}}$, results in a true derivative ($dA/d\lambda$). Integration of the difference trace yielded an approximation of the normal UV spectrum (Fig. 1, inset) of **2**. Thus, all UV binding studies used the amplitude of the difference spectrum, ΔA (Fig. 1), as a convenient and sensitive experimental measure of $\Delta\lambda_{\text{max}}$.

Binding studies with $\beta\text{-CD}_n$ were accomplished in an opposite mode to the above. The reference cuvette contained the polymer-treated (ca. $0\text{--}12 \text{ mM } \beta\text{-CD}$ monomer) solution of **2** in buffer, while the sample cell contained a buffered solution of **2** ($8 \times 10^{-5} \text{ M}$) without exposure to $\beta\text{-CD}_n$. In these experiments

spectral traces were similar to the normal UV spectra for **2**, but with absorbance varying proportionally to the sequestered species. Thus, our measured parameter, $\Delta A_{330\text{nm}}$, is the reduction in absorbance due to the removal of **2** by $\beta\text{-CD}_n$. CPMAS NMR spectroscopy (800 μs contact time) of $\beta\text{-CD}_n$ indicated that virtually all of the dry weight of the polymer was due to the CD moiety. Thus, all our calculations of K , with regard to $\beta\text{-CD}_n$, made the assumption that 97% of the dry weight of the polymer was due to the $\beta\text{-CD}$ monomer.

Apple juice experiments.—Ripe ‘Granny Smith’ apple fruits were cut into wedges and juiced with an Acme Supreme Juicerator. Enough ascorbic acid was added to the juice during extraction to produce a final concentration of ca. 2.9×10^{-4} M (50 ppm). Celite analytical filter aid was added (2%), and the juice was vacuum filtered through Whatman 541 filter paper. The clarified juice was then passed through a Falcon filter (0.45 μm) to remove particulates which carry PPO. HPLC analyses of apple juice used in the binding studies indicate the concentration of **2** (1.8×10^{-4} M) was ca. two-fold greater than that of (–)-epicatechin. In another apple cultivar, the molar ratio of **2**: (–)-epicatechin has been found to be as high as 1:1 ([CA] = ca. 2×10^{-4} M)⁸. Apple-juice binding data were collected with a Shimadzu UV160U spectrophotometer using the technique described in the above section (reference = juice; sample = juice + variable CD). The measurement of enzymatic browning inhibition has been published in detail previously¹. Briefly, relative inhibition of browning at time t was calculated as

$$\frac{\Delta R_{440\text{nm}}^{\text{control}} - \Delta R_{440\text{nm}}^{\text{treatment}}}{\Delta R_{440\text{nm}}^{\text{control}}};$$

where ΔR is the spectrophotometric reflectance change ($R_{\text{initial}} - R_t$) at 440 nm for either the treatment (+CD) or the control (–CD). All inhibition data were normalized relative to the highest value. Juices used for the browning studies were not filtered. In another experiment, 60 mL of unfiltered juice was stirred for 10 min with 20 mL of $\beta\text{-CD}_n$ slurry, the solution was allowed to settle, and the treated juice was decanted (time = 0 h). Either **2** or other PPO substrates (5 mg) were then added back to the $\beta\text{-CD}_n$ -treated juice at ca. 3 h into the experiment.

Calculation of K , ΔH^0 , and ΔS^0 .—Assuming a 1:1 stoichiometry

$$K = \frac{[\text{CD} \cdot \text{CA}]}{[\text{CD}][\text{CA}]}.$$

Expressing [CD] and [CA] in terms of initial concentrations, $[\text{CD}]_0$ and $[\text{CA}]_0$,

$$K = \frac{[\text{CD} \cdot \text{CA}]}{\{[\text{CD}]_0 - [\text{CD} \cdot \text{CA}]\} \{[\text{CA}]_0 - [\text{CD} \cdot \text{CA}]\}}.$$

If one observes a change in the chemical shift of some resonance on **2**

($|\delta_{\text{CA}} - \delta_{\text{CA}+\text{CD}}| = \Delta\delta_{\text{CA}}$), or ΔA using the UV technique, as a function of varying either $[\text{CD}]_0$ or $[\text{CA}]_0$,

$$K = \frac{[\text{CA}]_0 \frac{\Delta}{\Delta_{\text{max}}}}{\left\{ [\text{CD}]_0 - [\text{CA}]_0 \frac{\Delta}{\Delta_{\text{max}}} \right\} \left\{ [\text{CA}]_0 - [\text{CA}]_0 \frac{\Delta}{\Delta_{\text{max}}} \right\}};$$

where Δ represents either $\Delta\delta$ or ΔA . Conversely, observation of changes in δ_{CD} ($|\delta_{\text{CD}} - \delta_{\text{CD}+\text{CA}}| = \Delta\delta_{\text{CD}}$) as a function of varying either $[\text{CD}]_0$ or $[\text{CA}]_0$ yields

$$K = \frac{[\text{CD}]_0 \frac{\Delta}{\Delta_{\text{max}}}}{\left\{ [\text{CD}]_0 - [\text{CD}]_0 \frac{\Delta}{\Delta_{\text{max}}} \right\} \left\{ [\text{CA}]_0 - [\text{CD}]_0 \frac{\Delta}{\Delta_{\text{max}}} \right\}}.$$

Solving for Δ provides either

$$\left\{ \Delta_{\text{max}} \left(1 + [\text{CD}]_0 K + [\text{CA}]_0 K - \left[1 + 2[\text{CD}]_0 K + 2[\text{CA}]_0 K + [\text{CD}]_0^2 K^2 + [\text{CA}]_0^2 K^2 - 2[\text{CD}]_0 [\text{CA}]_0 K^2 \right]^{1/2} \right) \right\} \{ 2[\text{CA}]_0 K \}^{-1}$$

or the above with a denominator of $2[\text{CD}]_0 K$ for Δ_{CA} or Δ_{CD} , respectively. The parameters, ΔA_{max} or $\Delta\delta_{\text{max}}$ and K , were evaluated by fitting the experimental values of ΔA , $\Delta\delta_{\text{CA}}$ or $\Delta\delta_{\text{CD}}$ to the appropriate equation utilizing a modified Gauss–Newton¹¹ spreadsheet. Typical curve fits (ΔA versus $[\alpha\text{-CD}]_0$) for **2** at pH 3.6 (2 and 36°C) are shown in Fig. 2. Note that the small deviation of the experimental (Fig. 2, inset) from the calculated values of ΔA are indicative of an excellent fit. This method is more flexible than others^{15,16}, since either the guest or CD can be observed for spectroscopic perturbations as a function of variable CD or guest concentrations; however, the method is limited by the fact that the stoichiometric ratio must be 1 guest:1 CD.

The dependence of K on temperature (T) was derived from the Gibbs–Helmholtz equation¹⁷. Using this technique, standard enthalpy changes due to complexation (ΔH^0 s) were obtained from the slope of a plot of $\Delta G^0/T$ ($-R \ln K$) vs. $1/T$. Standard entropy changes due to complexation (ΔS^0 s) may be obtained from the slope ($\Delta S^0 = -\text{slope}$) of a plot of ΔG^0 vs. T or from the intercept ($\Delta S^0 = -\text{intercept}$) of the above relationship. In our calculations, ΔS^0 obtained by either of these methods resulted in identical estimates.

RESULTS AND DISCUSSION

Characterization of β -cyclodextrin · chlorogenic acid inclusion complex geometry and stoichiometry using NMR spectroscopy.—Chemical shifts of the ^1H NMR spectrum of **2** (100 mM) are given in Table I. In the presence of β -CD (11.25 mM),

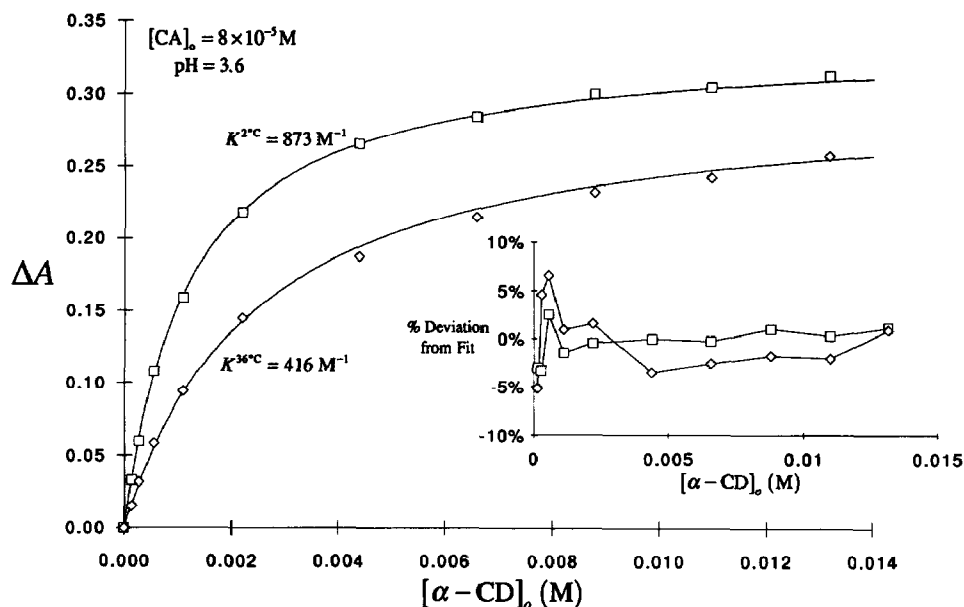


Fig. 2. Curve fits of ΔA as a function of $[\alpha\text{-CD}]_0$ for CA at pH 3.6 (2 and 36°C); inset, deviation of the experimental from the calculated values of ΔA .

the spectrum of **2** at 1.25 mM shows significant chemical-shift perturbations associated only with H-2', -5', and -6' ($\Delta\delta_{\text{CA}} = 36, 12$, and 49 Hz, respectively). As demonstrated previously for $\beta\text{-CD} \cdot (\text{S}) - (+)\text{-fenoprofen}$ complexation¹³, $\beta\text{-CD}$ (0.139 mM + 1.25 mM **2**) manifests the largest chemical shift changes at H-5 ($\Delta\delta_{\beta\text{-CD}}$ 33 Hz), probably because the most protuberant feature within the $\beta\text{-CD}$ cavity is a ring of these protons². Small $\Delta\delta_{\beta\text{-CD}}$ s were also observed for protons at positions 3 and 6 ($\Delta\delta$ 15 and 13 Hz, respectively). The largest $\Delta\delta_{\text{CA}}$ s were associated with protons on the benzene ring and suggest that this moiety docks within the $\beta\text{-CD}$ cavity. This binding geometry is similar to that hypothesized for the cinnamic acid $\cdot \text{CD}$ complex⁵. Other evidence for this binding geometry is that aromatic protons (H-2', -5', and -6') T_1 s were more perturbed by the binding process than protons adjacent (H-7', and -8') to this spin system (Table I). Since intramolecular relaxation is probably similar for **2** in either the free or bound state, and D_2O is the dominant species in our system, the change in T_1 due to complexation (ΔT_1) could result from intermolecular H–D relaxation (e.g., $\text{D}_2\text{O} \leftrightarrow \text{CA}$ protons 2', 5', and 6') where

$$\Delta T_1 \approx \frac{27\Delta D_{\text{trans}} \Delta r_{\text{IS}}}{16\pi \Delta c_s \gamma_I^2 \gamma_S^2 \{h/2\pi\}^2 S(S+1)}.$$

In the above equation¹⁸: c_s is the concentration of D per unit volume; D_{trans} , which is equal to

$$\frac{1}{2}(\text{D}_{\text{trans}}^{\text{CA}} + \text{D}_{\text{trans}}^{\text{D}_2\text{O}}),$$

TABLE I

¹H NMR assignments, chemical shifts, T_1 s, and ΔT_1 s of chlorogenic acid (CA, **2**) in the free and ca. 90% complexed states with β -cyclodextrin (β -CD)

Assignment	δ (ppm)	T_1 (ms)		ΔT_1 (ms)
		1.25 mM CA	1.25 mM CA + β -CD ^a	
6, 2 ^b	2.1, 2.0	350 \pm 23	301 \pm 16	–49
8'	6.1	660 \pm 21	638 \pm 12	–22
7'	7.4	835 \pm 31	843 \pm 19	8
2'	7.0	301 \pm 7	518 \pm 4	217
5'	6.3	331 \pm 6	606 \pm 6	275
6'	6.9	509 \pm 14	645 \pm 5	136

^a $[\beta\text{-CD}]_0 = 11.25$ mM; $K = \sim 800$ M^{–1} and $[\text{CA}]_0 = 1.25$ mM results in ca. 90% of CA bound. ^b T_1 s are calculated from the average intensity of CA's H-2 (axial-equatorial) and H-6 (axial-equatorial). All chemical shifts are reported for the uncomplexed state (100 mM).

is the mutual translational self-diffusion coefficient of **2** and D₂O that carry spins I and S, respectively; r_{IS} is the closest approach of spins I and S. Thus, the ΔT_1 s could result from any combination of c_s abating or D_{trans} and r_{IS} increasing.

The $\Delta\delta$ s of H-6' and H-5 resonances of **2** and β -CD, respectively, were monitored and, by applying the method of continuous variations (Job's plots: Fig. 3)^{12,13}, used to determine the stoichiometry of the β -CD · CA equilibrium of the inclusion complexes. In these experiments, the total concentration of all solutions was fixed at 10 mM, but the mole fractions, $\chi_{\beta\text{-CD}}$ and χ_{CA} , were varied from 0.1 to 0.9. Since each curve (Fig. 3) shows a maxima at $\chi = 0.5$, one may presume the

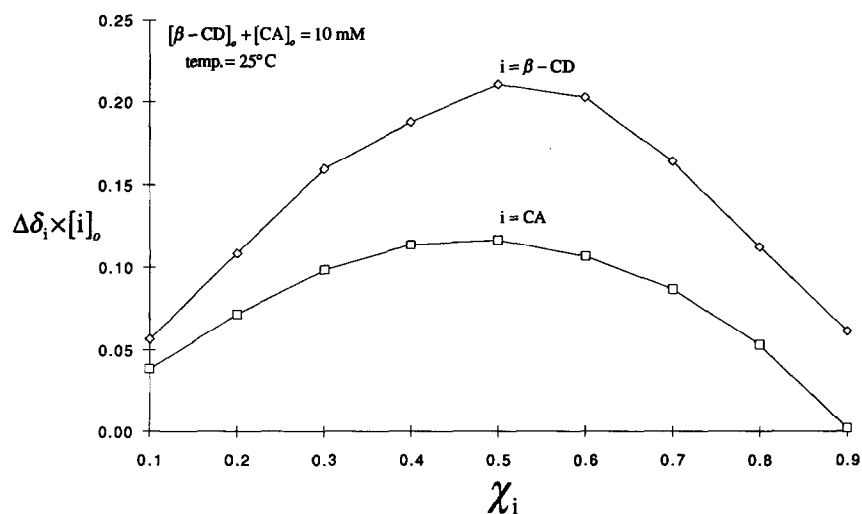


Fig. 3. Continuous variations plot of $\Delta\delta_{\text{CA}} \times [\text{CA}]_0$ (CA resonance H-6'), or $\Delta\delta_{\beta\text{-CD}} \times [\beta\text{-CD}]_0$ (β -CD resonance H-5), as a function of χ_{CA} or $\chi_{\beta\text{-CD}}$ at 25°C ($[\text{CA}]_0 + [\beta\text{-CD}]_0 = 10$ mM).

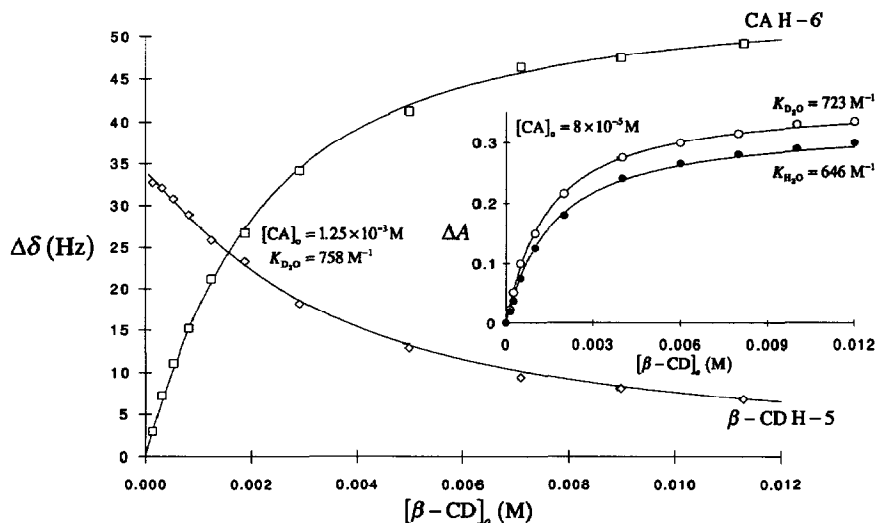


Fig. 4. Perturbation of ^1H NMR-derived $\Delta\delta_{\text{CA}}$ (H-6', open squares) and $\Delta\delta_{\beta\text{-CD}}$ (H-5, open diamonds) as a function of a variable $[\beta\text{-CD}]_0$ ($[\text{CA}]_0 = 1.25 \times 10^{-3}$ M) at 25°C in 0.05 M Na phosphate buffer (D_2O). Inset, changes in ΔA as a function of variable $[\beta\text{-CD}]_0$ ($[\text{CA}]_0 = 8 \times 10^{-5}$ M) at 25°C in 0.05 M Na Phosphate buffer (D_2O or H_2O).

presence of an equilibrium with the stoichiometry of 1 CA:1 β -CD, the most common ratio found for similar compounds in extensive literature reviews^{2,6,16}.

In order to calculate the apparent association constant, K , (Fig. 4), as described in the experimental section for a 1:1 stoichiometry, $\Delta\delta$ s of H-6' and H-5 resonances of **2** and β -CD, respectively, were measured as a function of variable $[\beta\text{-CD}]_0$ ($[\text{CA}]_0 = 1.25$ mM). From $\Delta\delta_{\text{CA H-6'}}$ (open squares) and $\Delta\delta_{\beta\text{-CD H-5}}$ (open diamonds) analyses, we obtained K s of $758 \pm 34 \text{ M}^{-1}$ and $758 \pm 23 \text{ M}^{-1}$ (\pm standard error), respectively, at 25°C in 0.05 M Na phosphate buffer in D_2O . Under these same conditions (Fig. 4, inset), utilizing the UV method described in the Experimental section, K was found to be $723 \pm 24 \text{ M}^{-1}$ (open circles). In order to determine the isotopic effect of D_2O , we repeated the UV experiment in H_2O and found K diminished to $646 \pm 33 \text{ M}^{-1}$ in 0.05 M Na phosphate buffer and to $597 \pm 27 \text{ M}^{-1}$ in 0.1 M buffer. These data show that K was approximately equal using either the NMR or UV method. However, by doubling the ionic strength and substituting H_2O for D_2O , K dropped by ca. 11 and 17%, respectively.

Inclusion complex thermodynamics as a function of CD type, pH, and ionic strength.—In Table II we have provided K s (from UV data) for α -CD, β -CD, $\beta\text{-CD}_n$, and γ -CD complexation with **2** as a function of variable temperature and pH. With the exception of $\beta\text{-CD}_n$, the K s for CA \cdot CD complexation decreased by ca. $50 \pm 14\%$ ($\bar{x} \pm s_x$) as the temperature increased from 3 to 37°C; ΔH^0 s were all negative, indicating that the complex dissociates when the temperature increases. β -CD displayed little sensitivity to pH with respect to K ; however, α -CD-associated K s increased $102 \pm 19\%$, and γ -CD-associated K s decreased $93 \pm 2\%$ as the

TABLE II

Apparent association constants (K), standard enthalpy (ΔH^0), and entropy (ΔS^0) change of CD·CA complexation as a function of temperature (t), pH, and cyclodextrin type

t (°C)	K (M ⁻¹)							
	α -CD		β -CD				γ -CD	
	pH 3.6	pH 6.5	Soluble		Polymeric		pH 3.6	pH 6.5
			pH 3.6	pH 6.5	pH 3.6	pH 6.5		
3	509 ± 14	1144 ± 22	873 ± 20	799 ± 23	332 ± 14	197 ± 18	555 ± 52	46 ± 9
13	426 ± 13	887 ± 33	672 ± 16	663 ± 21	428 ± 13	422 ± 42	412 ± 69	31 ± 6
25	321 ± 8	626 ± 23	526 ± 26	597 ± 27	501 ± 20	544 ± 34	400 ± 76	16 ± 8
37	249 ± 7	446 ± 10	416 ± 33	468 ± 19	499 ± 17	570 ± 32		
40					509 ± 19	593 ± 48		
50					360 ± 16	552 ± 47		
60					294 ± 14	330 ± 29		
ΔH^0 (3–37°C) ^a	-15.71	-20.43	-15.31	-11.41	11.74 ^b	27.98 ^b	-10.4	-30.34
ΔS^0 (3–37°C)	-4.74	-15.06	0.61	14.32	91.11 ^b	146.53 ^b	14.57	-78.15
ΔH^0 (25–40°C)					0.43	4.21		
ΔS^0 (25–40°C)					53.12	66.43		
ΔH^0 (40–60°C)					-24.45	-26.53		
ΔS^0 (40–60°C)					-26.34	-30.81		

^a ΔH^0 units in kJ mol⁻¹; ΔS^0 units in J mol⁻¹ K⁻¹. ^b Calculated from 3–25°C.

pH was changed from 3.6 to 6.5. A similar pH dependency has been shown before by other workers using flow microcalorimetry for β - and α -CD·*p*-nitrophenol complexation¹⁹. Probably due to its insoluble character, β -CD_{*n*}·CA displayed an unusual K -temperature dependency. At either pH 3.6 or 6.5, the K s increased substantially from 3 to 25°C, leveled off between 25 and 40°C, and decreased between 40 and 60°C. Thus, in the temperature range of 0–25°C, one observes a large positive ΔH^0 for the β -CD_{*n*}·CA equilibrium. This is somewhat unique since even small positive ΔH^0 s have been infrequently found in the cyclodextrin literature^{2,20}.

Enthalpy-entropy compensation²¹ is a term utilized to describe the behavior of ΔH^0 and ΔS^0 for a series of similar reactions (e.g., argon dissolving into solvent mixtures of different polarity, small ligands binding to proteins, etc.) driven by changes in H₂O solvation. In processes such as CD·CA interactions, where changes in the solvation of both guest and host play an important role in determining inclusion complex stability², a plot of ΔH^0 vs. ΔS^0 should result in a straight line with a slope $\Delta\Delta H^0/\Delta\Delta S^0$, known as the compensation temperature (T_c), of 270–320 K^{2,21}. Fig. 5 shows all our calculated values (Table II) of ΔH^0 plotted against ΔS^0 for β -CD·CA and β -CD_{*n*}·CA complexes (open circles) alongside data from numerous other β -CD·guest complexes² (closed circles). In this figure the solid line represents the best fit for all non-CA guest· β -CD interactions ($T_c = 330$ K); the inset of Fig. 5 ($T_c = 305$ K) represent our data alone. It is interesting that the β -CD_{*n*}·CA thermodynamics data show a trend, where-

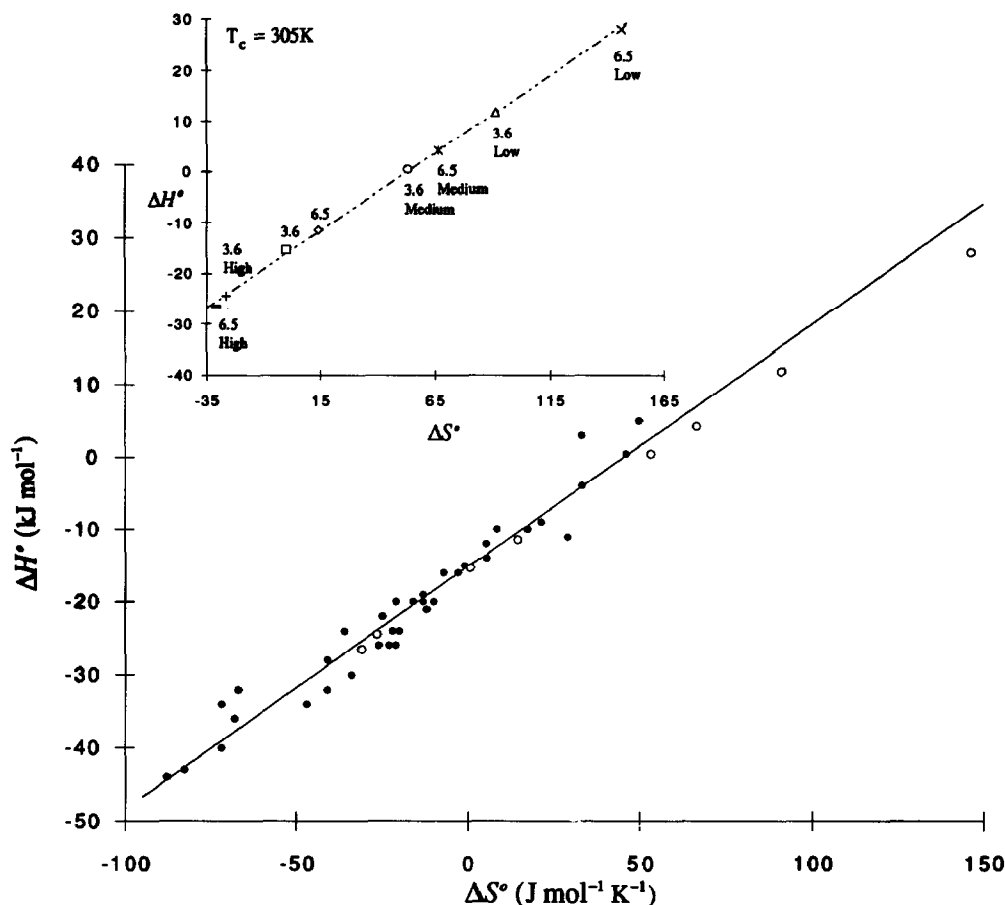


Fig. 5. Enthalpy–entropy compensation plots for β -CD and β -CD $_n$ ·CA inclusion complexes (open circles and inset). Data from other β -CD inclusion complex studies (closed circles) are provided for comparison.

upon the high temperature ranges (40–60°C) displayed the smallest ΔH^0 and ΔS^0 , followed by the medium (25–40°C) and the low (3–25°C) temperature ranges. Such enthalpy–entropy compensation behavior for the β -CD $_n$ ·CA series is probably related to temperature-dependent changes in H₂O solvation of the complex.

Water activity ($a_{\text{H}_2\text{O}}$) has been shown to have substantial effects on CD binding of azo dyes¹⁵ and *p*-nitrophenol¹⁹, whereupon the addition of Me₂SO to aqueous solutions drastically diminished K , presumably by decreasing $a_{\text{H}_2\text{O}}$. For the β -CD·CA equilibrium we found (Fig. 4 inset and Table II) $K_{\text{D}_2\text{O}} = 723 \text{ M}^{-1}$ and $K_{\text{H}_2\text{O}} = 646 \text{ M}^{-1}$ (0.05 M Na phosphate). Upon increasing the ionic strength, $K_{\text{H}_2\text{O}}$ decreased to 597 M^{-1} (0.1 M Na phosphate). In a more complete study (Fig. 6), we varied the ionic strength ($I = 0.07$ and 1.4 molal),

$$I = \frac{1}{2} \sum_i m_i Z_i^2,$$

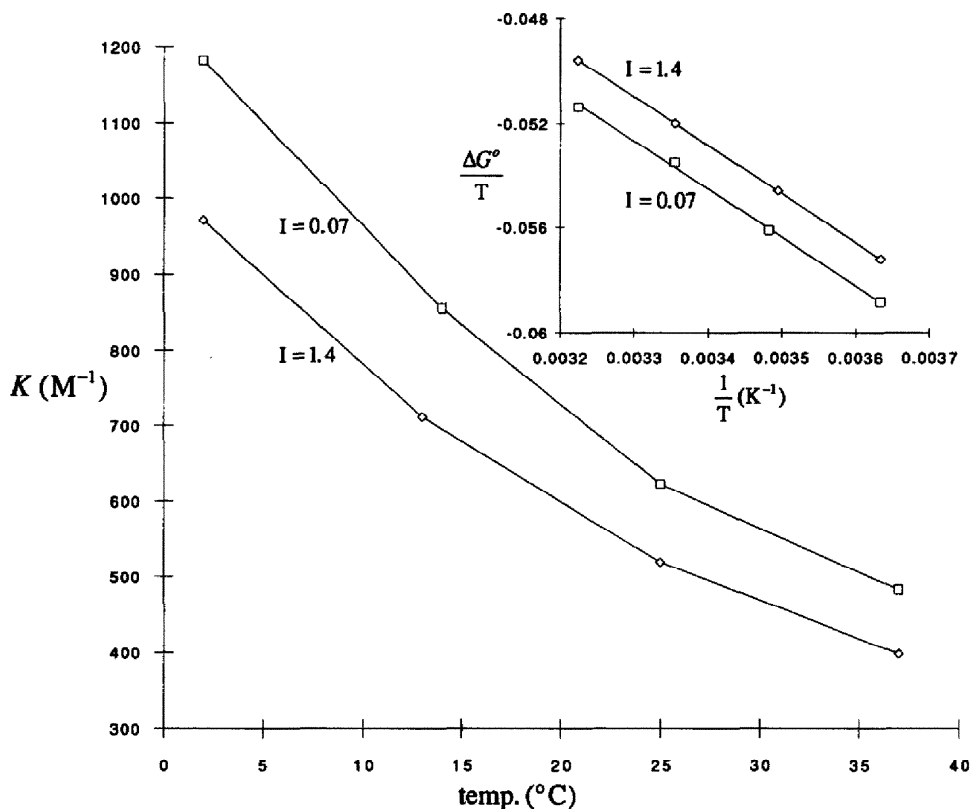
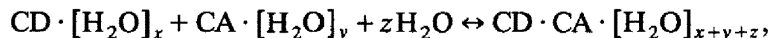


Fig. 6. Effect of Na phosphate buffer ionic strength (I , molality) on K as a function of temperature; inset, same data plotted as $\Delta G^{\circ}/T$ ($\text{J mol}^{-1} \text{K}^{-1}$) vs. T^{-1} (K^{-1}); slope = ΔH° ; intercept = $-\Delta S^{\circ}$.

of Na phosphate buffer by a factor of 20 (m is the molality and Z the charge). Regardless of temperature, K s were significantly smaller for the 1.4 molal treatment; T_c (298 K) calculated from these data was similar to T_c discussed previously (305 K). Assuming the major effect of increasing ionic strength or Me_2SO is to decrease $a_{\text{H}_2\text{O}}$, these data argue that there is a net increase in bound water in the complex over that found in CDs and guests alone. All the above support the following equilibrium



proposed previously¹⁵, where K is defined as

$$K = K^{\ddagger} \times a_{\text{H}_2\text{O}}^z;$$

in this relationship K , the apparent association constant, is equivalent to the association constant, K^{\ddagger} , times $a_{\text{H}_2\text{O}}$ to the power of the stoichiometric coefficient, z . The solvent isotope effect on K (Fig. 4, inset) argues for this model. Evidence against this equilibrium is the fact that the aromatic protons of **2** have larger T_1 s in the complexed than in the free state, which might indicate a lower D_2O concentra-

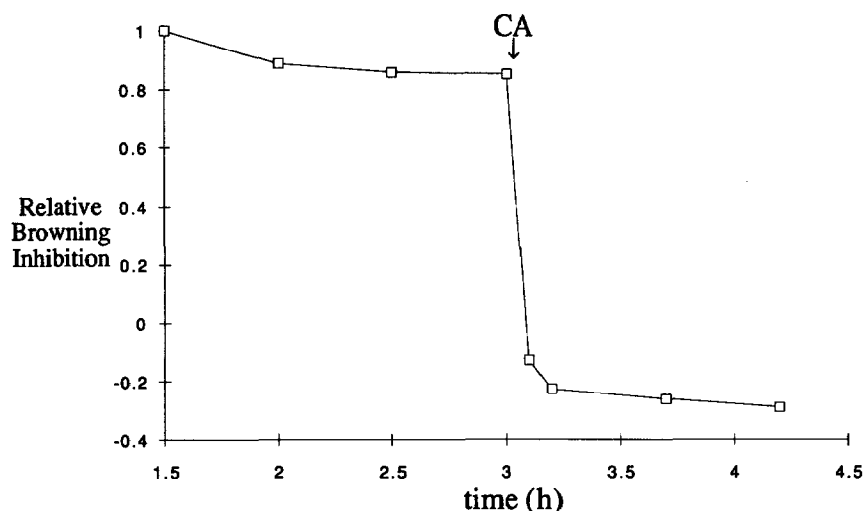


Fig. 7. Effect of added CA on browning inhibition of β -CD_n-treated apple juice as a function of time (h) at ca. 25°C.

tion near the aromatic protons (e.g., $\Delta c_s \downarrow$ and $\Delta r_{IS} \uparrow$); however, other relaxation mechanisms may be involved. Using Me₂SO to modify a_{H_2O} , Gerasimowicz and Wojcik¹⁵ have calculated that z ,

$$\ln K = \ln K^\ddagger + z \ln [H_2O],$$

was ca. 4–9 for several azo dyes. However, $Ks^{2,22}$ of the other guests have been shown to increase with ionic strength. Clearly, the CD binding mechanism is not simple², and variations in K due to ionic strength or Me₂SO are probably influenced by differences in a number of relatively weak interactions¹⁵ and not just a_{H_2O} .

Mechanism of the CD effect on the inhibition of PPO activity.—Enzymatic browning of raw fruits and vegetable products used in food service applications presents a difficulty for the processing industry due to the mandated restriction on sulfite utilization. Much effort has gone into discovering alternative to sulfites such as various antioxidants and their derivatives. Another approach has been to utilize chelating agents which arrest PPO activity, presumably by removing Cu²⁺, an important prosthetic group of the enzyme. The CDs studied in this work do, to varying degrees, inhibit enzymatic browning in apple fruit juice^{1,3}; however, nothing has been published about the mechanism by which CDs inhibit enzymatic browning. The most reasonable chemical mechanisms appear to be either sequestration of PPO substrates, such as CA (ref. 23), or the binding of the Cu(II) (ref. 24) of PPO by the chelating agent EDTA.

If the mechanism of enzymatic browning inhibition due to various CDs is related to the removal of PPO substrates, then juices treated with excess β -CD_n should initially show little color change (inhibition ~ 1), but should respond quickly

TABLE III

Qualitative browning response of β -CD_n-treated apple juice 4 h after treatment and apparent association constants with respect to β -CD at 25°C (0.1 M phosphate buffer)

Compound	Browning	$K_{\beta}^{25^{\circ}\text{C}, \text{pH } 6.5}$
2,6-Dihydroxybenzoic acid	—	—
2,5-Dihydroxybenzoic acid	—	—
3,4-Dihydroxybenzoic acid	+	$264 \pm 29 \text{ M}^{-1}$
L-Tyrosine	—	—
(-)-Epicatechin	+	$524 \pm 33 \text{ M}^{-1}$
Chlorogenic acid	+	$526 \pm 26 \text{ M}^{-1}$
Tryptophan	—	—
Rutin	—	—

to an added substrate such as **2** (inhibition ≤ 0) and turn brown. When unfiltered apple juice (see Experimental) was treated with β -CD_n (Fig. 7), no browning occurred until 5 mg of **2** was added 3 h into the experiment, whereupon browning occurred rapidly. Other PPO substrates were qualitatively tested (Table III) for activity. Of these compounds only **2** and (-)-epicatechin have been found in apple cider or apple extracts^{7,8}. Utilizing apple juice, which was processed to remove PPO (see Experimental), we performed UV-binding studies similar to those accomplished with **2**; however, instead of buffer + a known concentration of **2**, we

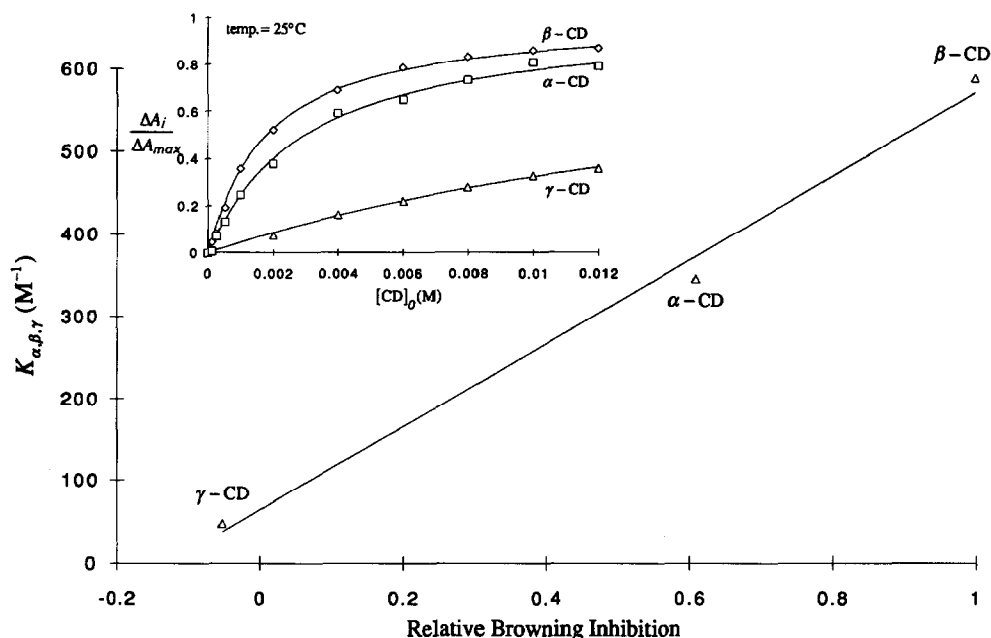


Fig. 8. Correlation K_s ($K_{\beta\text{-CD}} = 683 \pm 35 \text{ M}^{-1}$; $K_{\alpha\text{-CD}} = 379 \pm 37 \text{ M}^{-1}$; $K_{\gamma\text{-CD}} = 49 \pm 8 \text{ M}^{-1}$) with apple juice inhibition data³ (juice \pm ca. 6 mM CD; 25°C); normalized binding curves of filtered apple juice as a function of CD type.

used the processed juice (Fig. 8, inset) as the test medium. The K s we calculated ($K_{\beta\text{-CD}} = 587 \pm 35 \text{ M}^{-1}$; $K_{\alpha\text{-CD}} = 345 \pm 37 \text{ M}^{-1}$; $K_{\gamma\text{-CD}} = 48 \pm 8 \text{ M}^{-1}$) correlated well (Fig. 8) with inhibition data³ (juice \pm ca. 6 mM CD; 25°C) which were the average of 2 ΔR measurements (0.5 and 1 h after addition of CDs). The $K_{\beta\text{-CD}}$ and $K_{\alpha\text{-CD}}$ for apple juice (pH 3–4) were similar to K s (Table II) for the pH 3.6 model system; however, $K_{\gamma\text{-CD}}$ had a value more like the pH 6.5 model. All these experiments (Fig. 7, Table III and Fig. 8) argue that the mechanism for inhibition of juice browning with cyclodextrins is predominantly due to the binding of PPO substrates and not enzyme inactivation via sequestration of Cu^{2+} .

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