Journal of Agricultural and Food Chemistry

APRIL 1990
VOLUME 38, NUMBER 4

Quercetin Glucosides and Galactosides: Substrates and Inhibitors of Apple β-Galactosidase

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Quercetin 3-*O*-β-D-glucoside and -galactoside, 7-*O*-β-D-glucoside and -galactoside, and 3,7-di-*O*-β-D-glucoside and -galactoside were synthesized in improved yields by established methods. The last named compound, not previously described, was characterized by ^{1}H and ^{13}C NMR. The major β-galactosidase of apple fruit hydrolyzed the 7-galactosyl group but not the 3-galactosyl or any glucosyl group from the quercetin glycosides. The apple β-galactosidase was inhibited to a similar extent by quercetin and its 7-glycosides. Quercetin 3-glycosides were somewhat more effective inhibitors, and the 3,7-digalactoside was 25 times more effective ($K_i = 10^{-5}$ M). The strong inhibition of the β-galactosidase was not accounted for by the substrate properties of quercetin 3,7-digalactoside.

Structural aspects of the inhibition of enzymes by phenolic compounds are receiving increased attention (Rossi et al., 1986; Cody et al., 1986). A suggested structure–activity relationship of the inhibition of β-galactosidase of apple by flavonoids and other polyphenols (Dick and Bearne, 1988) was limited by the availability of suitable phenolic compounds. It was also desirable to find more effective phenolic inhibitors of this and related enzymes (Dick et al., 1990) in order to devise affinity methods for their isolation. A large variety of individual flavone and flavonol glycosides is not commercially available but these compounds are generally prevalent in plant epidermal tissue. Experimental quantities of these substances may be obtained from natural sources such as apple peel (Dick et al., 1987). However, quercetin 3-glucoside and quercetin 3-galactoside, which often occur together in plant tissue, are difficult to separate, and quantities of the individual compounds are more readily obtained by synthesis (Farkas et al., 1974). The contribution of the glycosyl configurations of these compounds as potential substrates and inhibitors of the apple β-galactosidase was of particular interest. The corresponding 7-glycosides of quercetin (Farkas et al., 1969) and the 3,7-diglycosides (Farkas et al., 1974) were synthesized as well in order to study the effect of glycosyl substituent position and of increased water solubility on their substrate and inhibitor properties with respect to the β-galactosidase.

MATERIALS AND METHODS

General Procedures. Solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade. Other chemicals were of reagent grade or better and were used without purification unless noted otherwise. Melting points were obtained on a Thomas-Hoover apparatus and are uncorrected. Proton and carbon-13 NMR spectroscopy was performed on a Nicolet NT-360 superconducting NMR spectrometer using hexadeuteriodimethyl sulfoxide as solvent. Fluorometric determinations were performed on a Kratos Fluoromat FS 950 fluorometer using an excitation filter of 365 nm and an emission filter of 435 nm. Polyamide 6S for column chromatography was a product of Riedel-DeHaen AG Seelze-Hannover. HPLC was performed on a Varian 5000 chromatograph with a Waters Radial Pak C_{18} reverse-phase column. Column effluent was monitored with a Varian UV50 detector, the output from which was analyzed with a Waters 840 chromatography data station. The solvent program was a gradient of tetrahydrofuran concentration (v/v) in 0.1% aqueous trifluoroacetic acid: 20–25% over 5 min, 25–60% from 5 to 10 min, 60% for 5 min. Retention times (min) of the synthetic quercetin glycosides in this system were as follows: quercetin 3,7-digalactoside, 2.48 ± 0.01; quercetin 3,7-diglucoside, 2.59 ± 0.02; quercetin 3-galactoside, 6.25 ± 0.04; quercetin 3-glucoside, 6.25 ± 0.03; quercetin 7-galactoside, 6.5 ± 0.2; quercetin 7-glucoside, 7.0 ± 0.2; quercetin, 13.16 ± 0.08.

Quercetin 3-*O*-β-D-Glucopyranoside (V). IV (Jurd, 1972; 2.76 g) was suspended in freshly distilled quinoline (7.5 mL) in a mortar. Tetra-*O*-acetylglucosyl bromide (Barczai-Martos and Korosy, 1950; 6.60 g) and dry silver(I) oxide (2.00 g) were added,

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the slurry was mixed with a pestle, and the mixture was placed in a vacuum desiccator at 0.05 atm for 90 min. The mixture was dispersed in glacial acetic acid (150 mL), and the suspension was centrifuged at 10000*g* for 15 min. The supernatant was poured onto crushed ice–water (2 L). After 30 min, the precipitate was collected by filtration, washed with water, and recrystallized from ethanol to give a cream-colored product (3.21 g). This acylated quercetin glucoside (2.00 g) was saponified under nitrogen in methanol/water (3/2, 250 mL) with 0.25 mol of NaOH for 1 h at 25 °C. After neutralization with HCl, the mixture was filtered and rotary evaporated in vacuo to dryness. The residue was suspended in water, heated to boiling for 20 min, filtered, and cooled, and the filtrate was made 0.1% with acetic acid and applied to a polyamide column (2.5 × 80 cm) equilibrated with 0.1% acetic acid. The column was irrigated successively with 0.1% aqueous acetic acid (1 L), 30% methanol in 0.1% aqueous acetic acid (1 L), a linear gradient (30–70%) of methanol in aqueous 0.1% acetic acid (1 L), and 70% methanol in aqueous 0.1% acetic acid. Column fractions (20 mL) were monitored by TLC on silica gel using ethyl acetate/acetone/water/acetic acid (6:3:1:1 (v/v)). Fractions containing V were pooled and reduced to dryness under reduced pressure. V was recrystallized from water to give fine yellow needles (0.28 g, 28%), mp 225–228 °C [Farkas et al. (1974), mp 238–242 °C]: $[\alpha]^{20}_D$ −37.2° (*c* 0.45, pyridine) [Horhammer et al. (1968), $[\alpha]^{20}_D$ −38.5°]; UV–vis (Markham, 1982), proton NMR, and carbon-13 NMR (Markham and Chari, 1982) spectra were as reported.

Quercetin 3-*O*-β-D-Galactoside (VI). VI was prepared from IV (2.76 g) and tetra-*O*-acetylgalactosyl bromide (6.60 g) as described for V. Recrystallization of the acylated quercetin galactoside from ethanol gave cream-colored crystals (3.03 g). Saponification of a sample (2.0 g) and polyamide chromatography as for V followed by recrystallization from 40% ethanol (v/v) gave fine yellow needles (0.27 g, 23%), mp 233–235 °C [Horhammer et al. (1968), mp 232–233]: $[\alpha]^{20}_D$ −79.4° (*c* 0.44, pyridine) [Horhammer et al. (1968), $[\alpha]^{20}_D$ −80.02°]; UV–vis (Markham, 1982), proton NMR, and carbon-13 NMR (Markham and Chari, 1982) spectra were as reported.

Quercetin 7-*O*-β-D-Glucoside (II). To a solution of I [Farkas et al. (1969), 2.5 g] and tetra-*O*-acetylglucosyl bromide (8.0 g) in freshly distilled quinoline (30 mL) was added dry silver(I) oxide (2.0 g). The mixture was stirred for 5 h in a stoppered flask. The coupled product (3.1 g) was isolated as for V. Saponification of a sample (2.0 g) as for V followed by polyamide chromatography and recrystallization from 80% aqueous ethanol (v/v) gave fine yellow needles of II (0.31 g, 29%), mp 247–248 °C [Farkas et al. (1969), mp 249–250 °C]: $[\alpha]^{20}_D$ −61.8° (*c* 0.43, pyridine) [Farkas et al. (1969), $[\alpha]^{20}_D$ −62.6°]; UV–vis (Markham, 1982), proton NMR, and carbon-13 NMR (Markham and Chari, 1982) spectra were as reported.

Quercetin 7-*O*-β-D-Galactopyranoside (III). III was prepared from I (2.5 g) and tetra-*O*-acetylgalactosyl bromide (8.0 g) as for II. Recrystallization of III from 80% aqueous ethanol (v/v) gave fine yellow needles (0.30 g, 28%), mp 244–246 °C [Fujiwara et al. (1976), mp 243–244 °C]: $[\alpha]^{20}_D$ −58.5° (*c* 0.45, pyridine); UV–vis (Markham, 1982), proton NMR, and carbon-13 NMR (Markham and Chari, 1982) spectra were as reported.

Quercetin 3,7-Di-*O*-β-D-glucoside (VII). VII was prepared from IV (2.5 g) and tetra-*O*-acetylglucosyl bromide (8.0 g) as for II. Saponification of a sample (2.0 g) of the coupled product followed by polyamide chromatography and recrystallization from 95% aqueous ethanol (v/v) gave fine yellow needles of VII (0.20 g, 30%), mp 217–220 °C [Farkas et al. (1974), mp 219–222 °C]: $[\alpha]^{20}_D$ −78.5° (*c* 0.42, pyridine) [Farkas et al. (1974), $[\alpha]^{20}_D$ −63°]; UV–vis (Markham, 1982), proton NMR, and carbon-13 NMR (Markham and Chari, 1982) spectra were as reported.

Quercetin 3,7-Di-*O*-β-D-galactoside (VIII). VIII was prepared from IV (2.5 g) and tetra-*O*-acetylgalactosyl bromide (8.0 g) as for II. Saponification of a sample (2.0 g) of the coupled product followed by polyamide chromatography and recrystallization from 95% ethanol (v/v) gave fine yellow needles of VIII (0.19 g, 32%), mp 218–220 °C: $[\alpha]^{20}_D$ −43.6° (*c* 0.53, pyridine). The UV–vis spectrum was as expected (Markham, 1982). Proton and carbon-13 NMR spectra are shown in Figure 2.

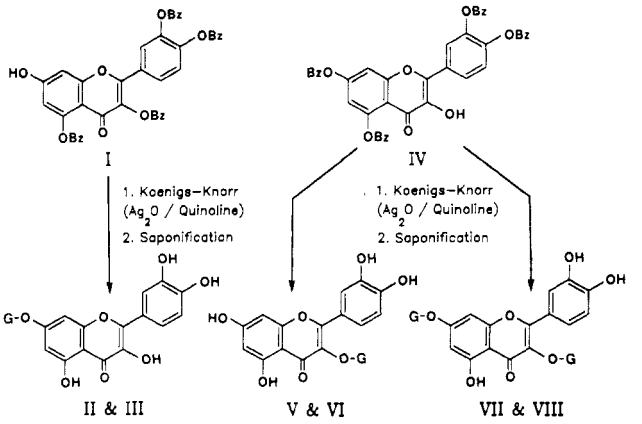

Figure 1. Coupling reactions (followed by saponification) of quercetin tetrabenzoates with acetobromohexoses to give quercetin 3-gluco-, 7-gluco-, and 3,7-diglucosides and quercetin 3-galacto-, 7-galacto-, and 3,7-digalactosides.

Apple β-Galactosidase. The enzyme was purified to apparent homogeneity by ion exchange and gel permeation HPLC (Dick et al., 1989). The enzyme preparation had a specific activity of 13.3 units/mg when assayed with 4-methylumbelliferyl β-D-galactoside (Dick and Bearne, 1988).

Hydrolysis of Quercetin Glycosides. Reaction mixture (2.5 mL) contained enzyme (1.8 × 10^{-4} unit) and quercetin glycoside (2.0 × 10^{-4} M) in 0.08 M acetate buffer, pH 4.0 at 30 °C. Samples (100 μL) were removed at 20-min intervals over a 5-h period for HPLC analysis. Product concentrations were determined by integration of HPLC peak areas.

β-Galactosidase Inhibition Assay. The procedure was patterned after Dick and Bearne (1988). Reaction mixtures (250 μL) contained enzyme preparation (5.9 × 10^{-5} unit), 0.05% BSA, 0.08 M sodium acetate buffer, pH 4.0, 4-methylumbelliferyl β-D-galactoside substrate (5.0 × 10^{-5} or 2.0 × 10^{-4} M), and quercetin glycoside inhibitor (a range of concentrations between 6.7 × 10^{-6} and 1.0 × 10^{-3} M). Reactions proceeded at 30 °C for 15 min and were terminated by addition of 4.0 mL of glycine buffer (0.15 M, pH 10.3). The product, 4-methylumbelliferone, was estimated fluorometrically.

RESULTS AND DISCUSSION

Synthesis of Quercetin Gluco- and Galactosides. Quercetin 7-*O*-β-D-glucoside and -galactoside (II and III) were synthesized from quercetin 3,3′,4′,5-tetrabenzoate (I) and the corresponding peracetyl-α-glycosyl bromide according to Farkas et al. (1969) (Figure 1). Quercetin 3-*O*-β-D-glucoside and -galactoside (V and VI) and quercetin 3,7-di-*O*-glucoside and -galactoside (VII and VIII) were prepared similarly from quercetin 3′,4′,5,7-tetrabenzoate (IV) (Farkas, 1974) (Figure 1). The yields of the 3-mono- and 3,7-di-*O*-glycosides, respectively, were improved considerably by adjustment of reactant ratio and reaction time (Materials and Methods). Each glycoside was readily purified on polyamide prior to crystallization and UV and NMR spectral characterization. Since quercetin 3,7-di-*O*-β-D-galactoside has previously not been described, its ^1H and ^{13}C NMR spectra are reproduced here (Figure 2). These substances are largely resolved and quantified in mixtures by reverse-phase HPLC (RP-HPLC) (Materials and Methods).

Hydrolysis by β-Galactosidase. Hydrolysis of the quercetin glycosides by the major β-galactosidase of apple fruit (Dick and Bearne, 1988) was monitored by RP-HPLC. The 7-β-galactosyl group from quercetin 7-*O*- and 3,7-di-*O*-β-D-galactoside was the only glycosyl group cleaved by the enzyme, quercetin and quercetin 3-*O*-β-D-galactoside, respectively, being the only products detected by RP-HPLC. The rate of hydrolysis of the monogalactoside was one-half while that of the digalactoside was

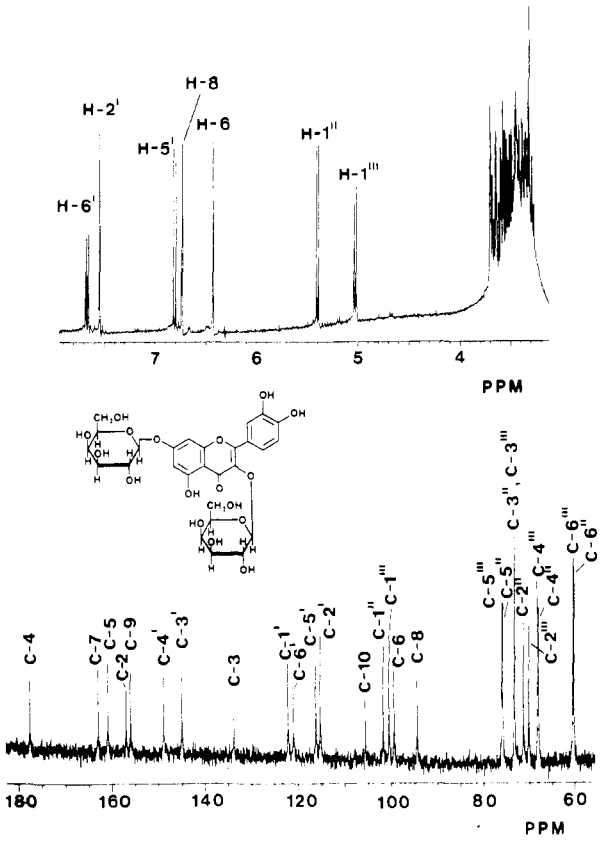

Figure 2. ^{1}H and ^{13}C NMR spectra of quercetin 3,7-di-*O*-β-D-galactoside.

equal to the rate of hydrolysis of 4-methylumbelliferyl β-D-galactoside, the standard substrate of the β-galactosidase. Quercetin 3-*O*-β-D-galactoside, one of the predominant quercetin glycosides present in apple epidermal tissue (Dick et al., 1987; McRae et al., 1989), was not hydrolyzed by the enzyme. This may account, partially, for the persistence of the galactosyl group at the 3 position and no other (Dick et al., 1990) of quercetin in this tissue.

β-Galactosidase Inhibition. The structure–activity profile of the inhibition of the major β-galactosidase of apple cortex tissue by flavonoids (Dick and Bearne, 1988) showed that 7-glycosylation of flavanones increased the inhibition significantly (e.g., naringin had a >10-fold smaller K_i value toward this enzyme than naringenin). The availability of the 7-glycosides of quercetin has now permitted the evaluation of this trend in flavones. Analysis of the inhibition by the method of Dixon of a highly purified preparation of the enzyme (Dick et al., 1989) by the quercetin glycosides synthesized herein showed that the inhibition was linear (e.g., Figure 3) and noncompetitive in each case (Table I). The noncompetitive inhibition indicates that inhibitor and substrate binding sites on the enzyme do not overlap, i.e., are separate. 7-Glycosylation of quercetin did not have the expected increased inhibitory effect (cf. naringin vs naringenin) in that the K_i value of the 7-glucoside or 7-galactoside (Table I) was essentially the same as that of quercetin (Dick and Bearne, 1988) itself. Apparently, binding of quercetin derivatives to the enzyme does not involve interaction of the 7-position of quercetin with the binding site. This interpretation is supported by the same K_i value for quercetin 3-*O*- and 3,7-di-*O*-glucosides. Presumably, the influence of 7-glycosylation of flavanones (Dick and Bearne, 1988) is conformational. 3-Glucosylation of quercetin increased its inhibition 2–3 times, somewhat better than the rhamnoside and rutinoside reported (Dick and Bearne, 1988). Galactosylation had the greatest effect. While the increased inhibition due to monogalactosylation might be explained by additional binding to the substrate binding site, not expected from the glucosides (the nature and precision of the kinetic analysis do not allow a distinction to be made between purely noncompetitive and a contribution from competitive inhibition), the very strong inhibition by the 3,7-digalactoside ($K_i = 10^{-5}$ M) was unique. It is unlikely that this great increase in inhibition was due to the digalactoside being an alternative substrate and therefore a competitive inhibitor of the enzyme because the 7-galactoside, also a substrate, did not show the same relative increase in inhibition compared to the corresponding glucoside. Also, K_m values (a rough guide to binding constants) for glycoside substrates of glycosidases (Dick et al., 1989) are generally 2 orders of magnitude higher. It would appear that the strong inhibition shown by the 3,7-digalactoside is due either to a unique binding site for it on the enzyme or to a unique conformation of the inhibitor that allows it to bind more strongly to the common binding site for flavonoids. X-ray crystallographic analysis (Cody et al., 1986) of the enzyme-inhibitor complexes would contribute to a resolution of the alternatives.

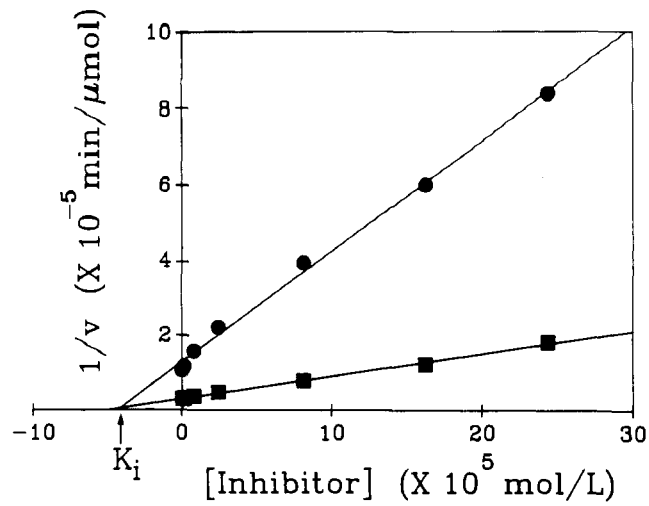

Figure 3. Dixon plot of the initial rate of hydrolysis of 4-methylumbelliferyl β-D-galactoside at 0.2 (■) and 0.05 mM (●) catalyzed by apple β-galactosidase in the presence of varying concentrations of quercetin 3-*O*-β-D-galactoside.

Table I. Inhibition Constants (K_i Values) of the Effect of Synthetic Quercetin Glycosides on the β-Galactosidase of Apple[a]

	app K_i, M × 10^4	
quercetin glycoside	0.2 mM substrate	0.05 mM substrate
3-glucoside	0.89 ± 0.08	1.12 ± 0.08
3-galactoside	0.52 ± 0.03	0.49 ± 0.02
7-glucoside	2.5 ± 0.2	2.4 ± 0.2
7-galactoside	1.6 ± 0.3	1.8 ± 0.2
3,7-diglucoside	0.89 ± 0.07	1.2 ± 0.1
3,7-digalactoside	0.10 ± 0.01	0.101 ± 0.004

[a] See Materials and Methods for conditions of inhibition assay.

ACKNOWLEDGMENT

We thank the Atlantic Region Magnetic Resonance Centre, Dalhousie University, Halifax, NS, Canada, and particularly Dr. T. B. Grindley for recording the NMR spectra and NSERC of Canada for an operating grant (A.J.D.).

LITERATURE CITED

Barczai-Martos, M.; Korosy, F. Preparation of Acetobromo-Sugars. *Nature* **1950**, *165*, 369.

Cody, V.; Middleton, E., Jr.; Harborne, J. B., Eds. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. Alan R. Liss: New York, 1986; pp 231-390.
Dick, A. J.; Bearne, S. L. Inhibition of β-Galactosidase of Apple by Flavonoids and Other Polyphenols. *J. Food Biochem.* **1988**, *12*, 97-108.
Dick, A. J.; Redden, P. R.; DeMarco, A. C.; Lidster, P. D.; Grindley, T. B. Flavonoid Glycosides of Spartan Apple Peel. *J. Agric. Food Chem.* **1987**, *35*, 529-531.
Dick, A. J.; Opoku-Gyamfua, A.; DeMarco, A. C. Glycosidases of Apple Fruit: A Multifunctional β-Galactosidase. Submitted for publication to *Physiol. Plant.*, **1990**.
Farkas, L.; Nogradi, M.; Vermes, B.; Wolfner, A.; Wagner, H.; Horhammer, L.; Kramer, H. Synthese des Quercemeritrins und Quercetin 3,7-diglucosids. *Chem. Ber.* **1969**, *102*, 2583.
Farkas, L.; Vermes, B.; Nogradi, M. Die Synthese Samtlicher Bekannter Naturlichen Quercetin 3,7-Bis(glucoside). *Chem. Ber.* **1974**, *107*, 1518.
Fujiwara, H.; Nonaka, G.; Vogi, A. Studies of the Components of the Leaves of *Coptis japonica* Makino. I. The Structures of Coptiside I and II. *Chem. Pharm. Bull.* **1976**, *24*, 407-413.
Horhammer, L.; Wagner, H.; Arndt, H.; Dirscherl, R.; Farkas, L. Synthese und Strukturbeweis von Isoquercitrin, Hyperosid und Quercetrin. *Chem. Ber.* **1968**, *101*, 450-453.
Jurd, L. Quercetin Derivatives. U.S. Patent 3 661 890, 1972.
Markham, K. R. Ultraviolet-visible Absorption Spectroscopy. In *Techniques of Flavonoid Identification*; Academic Press: London, 1982; p 39.
Markham, K. R.; Chari, V. M. Carbon-13 NMR Spectroscopy of Flavonoids. In *The Flavonoids Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Academic Press: New York, 1982; Chapter 2.
McRae, K. B.; Lidster, P. D.; DeMarco, A. C.; Dick, A. J. Comparison of the Polyphenol Profiles of Apple Cultivars by Correspondence Analysis. *J. Sci. Food Agric.* **1989**, in press.
Rossi, M.; Rickles, L. F.; Halpin, W. A. The Crystal and Molecular Structure of Quercetin: A Biologically Active and Naturally Occurring Flavonoid. *Bioorg. Chem.* **1986**, *14*, 55-69.

Received for review July 25, 1989. Accepted November 27, 1989.

Inhibition Studies on Apple Polyphenol Oxidase

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Inhibition studies by reductant, carboxylic acid, and halide compounds have been carried out on purified apple polyphenol oxidase (PPO). When ascorbic acid, cysteine, or bisulfite was added, a lag period was observed in the color formation whereas oxygen uptake was immediate. The most efficient compound in decreasing the remaining activity was bisulfite followed by cysteine and ascorbic acid. All tested aromatic carboxylic acids were pure competitive inhibitors. For a same substitution, inhibition decreased in the order cinnamic, benzoic, phenylpropionic, and phenylacetic. In each series, inhibition was slightly enhanced by *p*-hydroxy substitution and greatly decreased by *m*-methoxy substitution. Inhibition increased as pH was lowered from 5 to 3.6, and the results indicated that the neutral form of the carboxyl group was mainly responsible for inhibition. Sodium halides were inhibitors and the decreasing order was NaF, NaCl, NaBr, and NaI. When pH was varied between 3.5 and 5, fluoride exhibited the same behavior as the carboxylic inhibitors. The K_i for HF was close to 4 μM; thus it was by far the most potent inhibitor of apple PPO. Chloride was a noncompetitive inhibitor, and an equation is given for the variation of the apparent K_i of chloride with pH.

Enzymatic browning in fruits and vegetables is often an undesirable reaction, the prevention of which has always been a challenge to food scientists (Ponting, 1960). The oxidative browning of fruits is mainly due to polyphenol oxidase (EC 1.14.18.1; PPO). PPO has been the subject of recent reviews (Vamos-Vigyazo, 1981; Whitaker, 1985; Mayer, 1987). It is a copper enzyme that in the presence of oxygen catalyzes the oxidation of phenolic substrates in quinones, which then are polymerized to brown, red, or black pigments.

According to Mayer and Harel (1979), PPO inhibitors can be grouped into two classes: compounds that interact with copper in the enzyme and those that affect the active site for the phenolic substrate. Among the second type of inhibitors, aromatic carboxylic acids of the benzoic and cinnamic series have been widely studied since the first works of Kuttner and Wagreich (1953) and Krueger (1955). Although most authors found that these compounds were competitive inhibitors of PPO due to their structural similarities with phenolic substrates, some papers indicated that the type of inhibition was dependent on the substrate being used for assay and was either competitive, noncompetitive, or mixed (Soler-Martinez et al., 1965; McRae and Duggleby, 1968; Duckworth and Coleman, 1970; Rivas and Whitaker, 1973; Pifferi et al., 1974; Walker and Wilson, 1975; Walker and McCallion, 1980; Gunata et al., 1987). For the first type of inhibitors, inhibition by metal ion chelators such as azide (Healey and Strothkamp, 1981), cyanide (Duckworth and Coleman, 1970), and diethyldithiocarbamate, which are more or less specific for copper, as well as inhibition by inorganic halides has been studied for PPO from different sources (Krueger, 1955; Sharon and Mayer, 1967; Ben-Shalom et al., 1977; Penafiel et al., 1984; Martinez et al.,

0021-8561/90/1438-0926$02.50/0