Quercetin Glycosides and Chlorogenic Acid: Inhibitors of Apple β-Galactosidase and of Apple Softening

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Organic solvent extracts of Spartan apples, which inhibit β-galactosidase of apples and suppress apple softening in storage, have been fractionated by chromatography on silica gel and acrylic ester resin sheets and columns. The major constituents of the extracts, chlorogenic acid, catechins, and quercetin glycosides, individually inhibit polyphenoloxidase-free preparations of β-galactosidase and suppress softening of Golden Delicious apples held at 20 °C.

Organic solvent extracts of apples contain substances which inhibit the enzyme β-galactosidase in acetone–powder preparations of McIntosh apples (Dick et al., 1984). This enzyme has been implicated in cell-wall polysaccharide degradation in apple fruits (Knee, 1973; Bartley, 1977) although correlation of β-galactosidase activity with fruit firmness loss has not been established (Bérard et al., 1982; Dick et al., 1984). Since the enzyme is partially inhibited in most crude extracts of apples, it was suggested that inhibition of enzyme activity may be involved in the regulation of apple texture loss (Dick et al., 1984). The same organic extracts of apples, upon reapplication to Gravenstein and McIntosh apples, significantly retarded softening of these apples on the "shelf" (Lidster et al., 1985). This paper describes the preliminary fractionation and identification of the principal constituents of organic extracts of apples which are shown to individually inhibit β-galactosidase activity in vitro and of whole apple fruit firmness loss.

MATERIALS AND METHODS

Chlorogenic acid, (+)-catechin, (−)-epicatechin, quercetin, rutin, and methylumbelliferyl β-D-galactopyranoside were obtained from Sigma Chemical Co. All solvents used were of HPLC grade.

Extract Preparation. Ethyl acetate extracts of Spartan apples were obtained, concentrated, and purified by solvent partition as previously described (Lidster et al., 1985). The final syrupy residue was dissolved in H_2O for inhibition and reapplication experiments or in methanol or ethyl acetate for chromatographic analysis or further purification.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was carried out on Eastman silica gel sheets without fluorescent indicator. A typical solvent system used was 10% acetic acid in ethyl acetate in which the following R_f's were observed: chlorogenic acid, 0.06; rutin, 0.14; quercetin arabinoside, 0.4; catechins, 0.67. Purified ethyl acetate extracts (above) of apples upon TLC showed material with R_f 0.06, 0.67, and a broad zone of R_f 0.07–0.45. A variety of other solvents on TLC and HPLC (data not shown) confirmed the presence of chlorogenic acid and catechins. A solvent system which provided the greatest resolution on TLC of the components of the extract was dichloromethane, methanol, and acetic acid (84:15:1) in which catechins moved with the solvent front, chlorogenic acid remained almost at the origin, and quercetin glycosides had R_f's ranging from 0.1 to 0.7.

Flash Chromatography. Flash chromatography was performed on a 650-mL "Baker" column (4.0-cm diameter) with "Baker" silica gel for flash chromatography by the procedure described by Still (1978). A nitrogen gas pressure of 17.2 kPa was applied to obtain a linear flow rate of approximately 3 cm min^{-1}. Fractions (30 mL) collected were analyzed by TLC and assayed for inhibition of β-galactosidase. Extracts from 50 kg of apples were applied to a column equilibrated with dichloromethane, methanol, and acetic acid (84:15:1) and the column developed with 900 mL of this solvent. Combined fractions 7–21 from this column, which were enriched for quercetin glycosides but were still contaminated with catechins and chlorogenic acid, were concentrated, and were applied to a second column. This was developed with 900 mL of dichloromethane, methanol, and acetic acid (89:10:1). Fractions 18–21, which appeared most homogeneous as determined by TLC were combined. Upon standing in the cold overnight, a crystalline solid (0.2 g) was obtained which upon recrystallization from methanol, dichloromethane, and hexane had mp 206–210 °C.

Characterization of the Quercetin 3-Arabinoside Obtained by Flash Chromatography. The ultraviolet spectrum in methanol showed absorption maxima at 360 and 258 nm and the spectrum decomposed in sodium methoxide (Mabry et al., 1970). The ultraviolet spectrum was consistent with that of a quercetin glycoside. The electron impact mass spectrum (Williams, 1984) showed an intense peak at M/E 302 for the aglycone, and the molecular formula was deduced from the relative intensities of the M + 1 and M + 2 peaks by comparison with calculated values (Pavia et al., 1978). The chemical ionization mass spectrum (Williams, 1984) showed a major peak at M/E 303 which corresponds to the aglycone with a hydrogen atom transferred from the isobutane ionization gas. The EI spectrum corresponded well to the known fragmentation pattern of quercetin (Harborne et al., 1975). Proton NMR spectra were obtained with a Varian EM 360L Spectrometer with deuterated acetone as solvent and Me_4Si internal standard (Williams, 1984). The spectrum was comparable to published spectra of quercetin 3-glycosides (Mabry et al., 1970). The sugar was identified, by standard procedures of acid hydrolysis followed by paper chromatographic separation (Mabry et al., 1970), as arabinose. A trace of xylose was also detected. Quercetin was isolated from the hydrolysate in a crystalline state.

Isolation of Quercetin Glycoside Fraction. An acrylic ester resin (Amberlite XAD 7) column (40 × 2 cm) equilibrated with 30% methanol in aqueous 0.1 M acetic acid was used to fractionate a 10-g sample in 25 mL of the same solvent of purified ethyl acetate extract from Spartan apples (100 kg). The column effluent was monitored by

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TLC and absorption in the ultraviolet at 280 nm. Chlorogenic acid and catechins were eluted with four column volumes of the initial eluent. A linear gradient (2 L) of 30% methanol to 70% methanol in aqueous 0.1 M acetic acid was applied to elute the quercetin glycosides. Resolution of individual quercetin glycosides was not achieved on this column (data not shown). Fractions containing the bulk of the quercetin glycosides were combined and concentrated by evaporation to a dark orange syrup (5.2 g). The syrup was characterized by acid hydrolysis followed by HPLC analysis of quercetin (data not shown) and paper chromatography of the sugars present which revealed glucose, galactose, rhamnose, arabinose, and xylose in agreement with the data of Siegelman (1955).

Assay of β-Galactosidase Inhibition. β-Galactosidase, in a 0.1 M phosphate buffer (pH 7.0) extract (Williams, 1984) of acetone powders of Spartan apples (Dick et al., 1984) was used for monitoring the fractionation of ethyl acetate extracts of apples. These preparations gave only semiquantitative inhibition data because they contained polyphenoloxidase activity which caused the inhibitory effects to be time dependent. β-Galactosidase activity was assayed fluorimetrically with methylumbelliferyl-β-D-galactoside (Leaback, 1975). Standard assay conditions for measuring inhibition, established by Bearne (1985), were volume of reaction 0.5 mL, pH 4.0, substrate concentration 0.2 mM, amount of enzyme 1×10^{-4} units, 0–200 μg of putative inhibitor. After quenching the reaction with alkali, a Kratos Fluorometer (Model FS 950) was used to measure fluorescence (excitation 365 nm, emission 460 nm). One unit of β-galactosidase inhibitor present in the quercetin glycoside fraction was defined as that amount which caused 50% inhibition in the standard assay described above. Quantification of inhibition required the use of polyphenoloxidase-free preparations of β-galactosidase. This may be achieved by passing an extract of an acetone powder of apples through a DEAE-agarose column at low ionic strength and collection of the void volume fraction (Sheldon, 1985).

Application of Extract Fractions and Polyphenols to Apples. Storage Tests. Preclimacteric McIntosh and Gold Delicious apples (*Malus domestica* Borkh.) were harvested from each of 5 trees (replicates) in a commercial orchard in Kentville, N.S. All fruit (50 apples for each cultivar replicate) was either dipped in the crude extract (200 units/mL) fraction for 15 s or placed in glass desiccators in either H_2O or the crude extract (200 units/mL, Lidster et al., 1985), a vacuum (0.05 atm) drawn for 2.5 min, after which the vacuum was released, and equilibration to ambient atmosphere allowed for a further 0.5 min. All fruit were cooled to 0 °C within 24 h, placed in 38-μm perforated polyethylene bags to maintain humidity of 92–95% RH, and then held at 0° for the duration of the experiment. Ten fruit from each treatment–replicate were removed at 50-day intervals and fruit firmness determined on opposite pared sides of apples by using a Ballauf penetrometer with an 11.1-mm tip.

Authentic Inhibition Compound Effects on Fruit Softening at Shelf Temperatures. Golden Delicious apples which had been stored for 7 months were obtained from a commercial CA storage held at 3% CO_2 + 2.5% O_2 and 0 °C. Fruit from a single bulk bin (350 kg) was divided randomly into groups of 4 treatments × 5 shelf life durations × 5 replicates of 10 fruit each. All fruit were warmed to 20 °C and fruit for each treatment immersed either in H_2O or in solutions of 0.003% (w/v) quercetin, 0.1% (w/v) quercetin glycoside fraction (from acrylic ester resin column extracted from apples), or 0.1% (w/v) chlorogenic acid in glass desiccators and a vacuum drawn as previously described. Fruit were then held at 20 °C and 80% RH and 10 fruit were removed for each treatment–replicate at 3, 7, 10, 14, and 20 days. Fruit firmness was determined as previously described. All data for each apple experiment were subjected to an analysis of variance and the variance estimates presented as standard errors.

Table I. Inhibition of β-Galactosidase[a] of Apples by Polyphenols Found in Apples

inhibitor	μg of inhibitor required to cause 50% inhibition
chlorogenate	40
catechin	196
quercetin	43
quercetin glycoside fraction	50
quercetin arabinoside	50

[a] Polyphenoloxidase-free preparation of β-galactosidase.

RESULTS AND DISCUSSION

The principal chemical constituents of the ethyl acetate extract fraction which inhibits β-galactosidase of apples (Lidster et al., 1985) were identified as chlorogenic acid, catechins, and quercetin glycosides. They are effectively resolved by TLC and the identification of chlorogenic acid and catechins was established by comparison with authentic compounds (see Materials and Methods). The major presence of quercetin glycosides was established in two ways: (1) the isolation of a quercetin arabinoside by flash chromatography of the extract on silica gel and (2) the quantitative separation of a quercetin glycoside fraction from chlorogenic acid and catechins on an acrylic ester resin column.

Flash chromatography of the extract fraction on silica gel is not recommended because of the poor resolution of the components of the mixture despite the fact that the same solvent (dichloromethane, methanol, acetic acid, 84:15:1) performed well in TLC on silica gel. Nonetheless, repetition of the chromatogram provided a crystalline sample of quercetin arabinoside. Its structure was established by ultraviolet, NMR, mass spectrometric methods (Harborne et al., 1975; Mabry et al., 1970), and paper chromatographic analysis of a hydrolysate. The quercetin glycoside fraction from the acrylic ester resin column, characterized by chromatographic analysis of a hydrolysate, had residues of glucose, galactose, rhamnose, arabinose, and xylose in agreement with Siegelman (1955).

Flash chromatography column effluent fractions were monitored for the ability to inhibit β-galactosidase activity of acetone powder extracts of apples. All fractions which contained any of chlorogenic acid, catechins, or quercetin glycosides inhibited such preparations (Williams, 1984). However, the use of these β-galactosidase preparations to reveal inhibition of the enzyme were of semiquantitative value only since they contained polyphenoloxidase which increases the inhibitory potency of these compounds upon prolonged incubation.

A purified preparation of β-galactosidase, free of polyphenoloxidase, was used to quantify the effectiveness as enzyme inhibitors of the purified quercetin glycoside fraction, quercetin arabinoside, chlorogenic acid, and catechin. Table I shows the concentration of these substances required to reduce the activity of the enzyme by half, i.e., 50% inhibition. With the exception of catechin which is less effective, these compounds (at less than 1 mM) gave comparable levels of inhibition.

The application of the apple extract containing inhibitors of β-galactosidase either as a postharvest dip or a vacuum infusion to either McIntosh or Golden Delicious

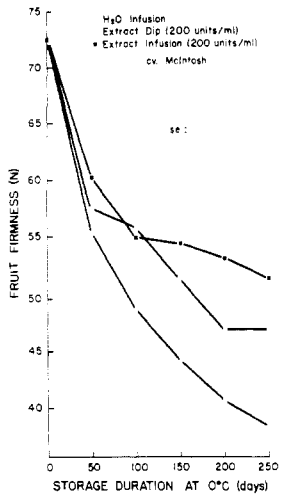

Figure 1. Effects of apple extract, containing β-galactosidase inhibitors, application to whole apples on firmness loss of McIntosh in 0 °C air storage.

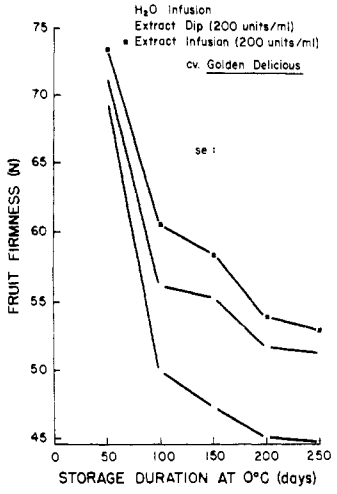

Figure 2. Effects of apple extract, containing β-galactosidase inhibitors, application to whole apples on firmness loss of Golden Delicious in 0 °C air storage.

apples suppressed firmness loss in storage as compared to that of the water infused controls (Figures 1 and 2). Vacuum infusion of the apple extract into whole fruit was more effective in maintaining apple firmness in storage than dipping, with the exception of a 100 day duration for McIntosh at which there was no significant difference between fruit firmness of dipped or vacuum infused apples. A comparable purified ethyl acetate extract fraction of Spartan apples was previously shown to retain fruit firmness in both preclimacteric Gravenstein and McIntosh apples held at 20 °C shelf temperature (Lidster et al., 1985). This extract was determined to specifically retard fruit firmness loss and did not modify the loss of titratable acids or soluble solids.

Vacuum infusion of pure 0.1% (w/v) chlorogenic acid or the 0.1% (w/v) quercetin glycoside fraction extracted and purified from apples significantly suppressed fruit firmness loss at 20° C shelf temperatures as compared to the firmness lost by the H_2O-infused controls (Figure 3). The application of quercetin 0.003% (w/v) retarded fruit softening as compared to the H_2O control at all durations except samples taken at 10 days. The lower concentration of quercetin, dictated by this compound's solubility in H_2O, was overall less effective in retarding fruit softening

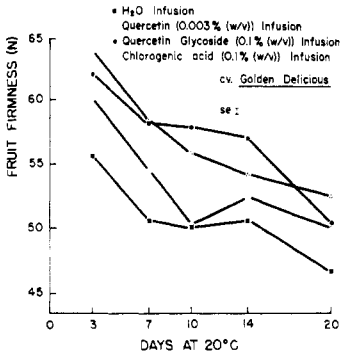

Figure 3. Effects of authentic inhibitor application to whole apples on firmness loss in Golden Delicious apples held at 20 °C.

than either chlorogenic acid or the quercetin glycoside fraction.

The present results confirm the suppression of fruit softening by naturally occurring compounds with β-galactosidase inhibitory properties (Dick et al., 1984). Several of the principal constituents were identified and some of these individual compounds showed the ability to suppress apple softening at shelf temperatures. Additional studies are required to assess the relative efficacy of the compounds and related metabolites identified in the apple extract on the suppression of whole fruit softening in cold storage and shelf temperatures.

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Registry No. β-galactosidase, 9031-11-2; chlorogenic acid, 327-97-9; (+)-catechin, 154-23-4; quercetin, 117-39-5; quercetin arabinoside, 30370-87-7; (−)-epicatechin, 490-46-0.

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