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Enzymatic Hydrolysis of Flavonoids and Pectic Oligosaccharides from Bergamot (*Citrus bergamia* Risso) Peel

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Pectinolytic and cellulolytic enzymes (Pectinase 62L, Pectinase 690L, and Cellulase CO13P) were used to evaluate the solubilization of carbohydrates and low molecular weight flavonoids from bergamot peel, a major byproduct of the essential oil industry. The enzymes were characterized for main-chain and side-chain polysaccharide hydrolyzing activities and also against pure samples of various flavonoids previously identified in bergamot peel to determine various glycosidase activities. The addition of Pectinase 62L or 690L alone, or the combination of Pectinase 62L and Cellulase CO13P, was capable of solubilizing between 70 and 80% of the bergamot peel, and up to 90% of the flavonoid glycosides present were cleaved to their aglycones. Cellulase CO13P alone solubilized 62% of the peel but had no deglycosylating effect on the flavonoid glycosides. Over a 24-h time course, a rapid release of cell wall carbohydrates was observed after treatment with Pectinase 62L, with a concurrent gradual hydrolysis of the flavonoid glycosides. Size-exclusion chromatography of the solubilized extract showed that after 24-h incubation, the majority of the solubilized carbohydrates were present as monosaccharides with a smaller proportion of oligosaccharides.

KEYWORDS: ***Citrus bergamia*; peel; byproducts; fungal hydrolytic enzymes; flavanone glycosides; oligosaccharides; bioavailability**

INTRODUCTION

Bergamot (*Citrus bergamia* Risso), a typical fruit of the Reggio Calabria province of Southern Italy, is used mostly for the extraction of its essential oil from the peel. Among the *Citrus* peel oils, because of its unique fragrance and freshness, bergamot oil is the most valuable and is therefore widely used in the cosmetic and food industries. Because of its antiseptic and antibacterial properties, the essence is also used in the pharmaceutical industry (*1*). Bergamot peel represents ∼60% of the total weight of the processed fruit and remains as a primary waste, causing many economic and environmental problems because of its fermentability. However, bergamot peel still contains potentially high value compounds, such as pectin and flavonoids (*2*).

Fungal glycosidases have been used for improving the bioavailability of flavonoids in two recent studies. In the first study, they have been used to treat fruit juices and tea to convert rhamnoglucosides (rutinosides and neohesperosides) to their corresponding glucosides which are more easily metabolized in humans and thus have greater bioavailability (*3*). In a second study, involving a human intervention experiment, it was clearly shown that pretreatment of hesperidin (hesperetin 7-*O*-rutinoside) in juices with a rhamnosidase greatly increased hesperetin bioavailability, by conversion to hesperetin 7-*O*-glucoside, as shown by increased blood plasma concentrations of total hesperetin (*4*). This type of partial hydrolysis can also be achieved by chemical means but is less suitable if the material is to be used for food supplements such as conversion of rhamnoglucosides to glucosides by refluxing with cyclohexanol and formic acid (*5*).

Pectins are complex polysaccharides which represent one of the major components of the plant cell wall of dicotyledonous plants, where they control the ionic status, cell expansion, and separation (*6*). They consist of "smooth regions": a backbone

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of α-(1→4)-linked galacturonic acid (GalA) residues which are partially methyl- or acetyl-esterified and of "hairy regions": a rhamnogalacturonan backbone where neutral sugar side chains, typically arabinan, galactan, and arabinogalactan, are attached (*7*). Pectins are commercially extracted from *Citrus* peel or apple pomace and are used in the food industry as natural thickeners and emulsifiers because of their rheological and gelling capacity (*8*, *9*). They are also applied as stabilizers in acid milk products and fat mimetics (*10*).

Flavonoids have been found to have health-related properties, including anticancer, antiviral, and anti-inflammatory activities (*11*). It is believed that they act as scavengers of free radicals, as well as modulate the activities of metabolic enzymes involved in the initiation of low-density lipoprotein oxidation (e.g., xanthine oxidase, glutathione reductase, lipoxygenase, and NADPH-oxidase) and inhibit cellular proliferation (*12*). In addition, they have been implicated in the defense of plants against invading pathogens, including bacteria, fungi, and viruses (*13*).

While chemical extraction of pectic-oligosaccharides and flavonoids from bergamot peel will lead to a high yield of recovery, enzymatic extraction allows further utilization of the residue and the extractable products, as well as provides a more environmentally friendly approach. In a recent paper, we showed that two fungal enzyme preparations (Pectinase 690L, previously referred to as Depol 690L, and Pectinase 62L) were able to solubilize a high percentage of bergamot peel, probably because of the release of pectinaceous material (*2*). Both pectinase preparations improve the yield from pressed fluid pulps by breaking down the structural pectins.

In this paper, pectinase preparations are characterized for a spectrum of activities and are evaluated for their ability to release pectic oligosaccharides and flavonoids from bergamot peel extracts, thus improving their bioactive properties.

MATERIALS AND METHODS

Source of Bergamot Peel. Bergamot peel was obtained from a bergamot processing factory (Consorzio del Bergamotto) in Reggio Calabria, Southern Italy. Fresh peel, consisting of a mix of the three major cultivars *Fantastico* (90%), *Femminello* (5%), and *Castagnaro* (5%), was divided into subsamples of approximately 500 g and was lyophilized.

Enzymes and Chemicals. Pectinase 690L (Endogalacturonase 190.7 U/mL, Cellulase 188.9 U/mL from *Aspergillus* sp. and *Trichoderma* sp.), Pectinase 62L (Endogalacturonase 1060.0 U/mL from *Aspergillus* sp.), and Cellulase C013P (45.6 U/mL of a 10 mg solid/mL solution from *Trichoderma* sp.) (**Table 1**) were obtained from Biocatalysts Ltd (Cefn Coed, Wales, United Kingdom). Wheat arabinoxylan (medium viscosity) and rye arabinoxylan were purchased from Megazyme International Ireland Ltd (Bray, Ireland). Birchwood xylan was obtained from Roth (Karlsruhe, Germany). Oat spelt xylan was purchased from Fluka (Dorset, United Kingdom). All *p*-nitrophenyl (pNP) substrates, potato arabinogalactan, carboxymethylcellulose, orange peel polygalacturonic acid (pectin), and rutin were obtained from Sigma Chemical Co (Dorset, United Kingdom). Sugar beet arabinan was a kind gift from British Sugar (Peterborough, United Kingdom). All flavone and flavanone glycoside substrates and aglycones used in the enzyme studies and for identification purposes were of HPLC grade of purity >95% and were obtained from Extrasynthèse (Genay, France). Solvents used were HPLC grade and water was ultrapure (distilled and deionized).

Enzyme Assays. Polygalacturonase (PGase), xylanase, arabinanase, galactanase, and cellulase activity were determined by measuring the enzymatic release of reducing groups at 580 nm from their respective substrates: orange peel pectin, birchwood and oat spelt xylan, wheat and rye arabinoxylan, sugar beet arabinan, arabinogalactan, and carboxymethyl cellulose (1%, w/v) by the dinitrosalicylic acid method previously described for xylanase (*2*). One unit of activity was defined as the amount releasing 1 μmol of sugar per min at 37 °C and pH 5.0. The release of *p*-nitrophenol (pNP) from pNP-glycosides (**Table 1**) was determined (modification of (*14*), using 1 mM substrate in 50 mM Na-acetate buffer, pH 5.0). The ability to deglycosidate flavonoids was determined using individual pure flavonoid glycosides: rutin (quercetin 3-*O*-rutinoside), naringenin 7-*O*-glucoside, naringin (naringenin 7-*O*-neohesperoside), eriodictyol 7-*O*-glucoside, neoeriocitrin (eriodictyol 7-*O*-neohesperoside), and hesperidin (hesperetin 7-*O*-rutinoside). Each substrate was added to give a final concentration of 200 μM in 1 mL 50 mM Na-acetate buffer, pH 5.0, and 50 μL of the undiluted commercial Pectinase 62L or Pectinase 690L was added to initiate the reactions. Samples were incubated for 2 h at 37 °C, and the reactions were terminated by addition of 1050 μL of 0.1% trifluoroacetic acid in 100% methanol. The samples were vortex mixed and then were centrifuged (17 000*g*, 4 °C, 10 min) to remove insoluble material. Samples were analyzed using the HPLC method previously described (*2*).

Table 1. Activity Profiles of the Enzyme Preparations Used in This Study[a]

substrate	activity (U/mL) Pectinase 690L	Pectinase 62L	Cellulase C013P
polygalacturonic acid	190.7 (±2.9)	1060.0 (±8.2)	–
carboxymethyl cellulose	188.9 (±2.5)	–	45.6 (±1.5)
arabinan	16.8 (±0.2)	17.1 (±0.5)	–
arabinogalactan	5.7 (±0.02)	20.2 (±2.2)	0.79 (±0.002)
oat spelt xylan	81.8 (±2.3)	29.3 (±1.1)	5.2 (±0.9)
birchwood xylan	80.2 (±3.0)	28.8 (±1.2)	3.5 (±0.6)
wheat arabinoxylan	81.4 (±2.6)	27.5 (±1.3)	4.1 (±0.5)
rye arabinoxylan	82.5 (±1.4)	31.7 (±1.0)	5.4 (±0.8)
pNP-β-galactopyranoside	0.9 (±0.02)	5.3 (±0.1)	0.006 (±0.0004)
pNP α-arabinofuranoside	1.5 (±0.05)	3.2 (±0.08)	0.02 (±0.006)
pNP α-rhamnopyranoside	0.01 (±0.0004)	0.08 (±0.001)	–
pNP α-glucopyranoside	0.02 (±0.001)	0.05 (±0.004)	–
pNP β-glucopyranoside	1.3 (±0.023)	0.8 (±0.02)	0.09 (±0.004)
pNP β-xylopyranoside	0.04 (±0.002)	0.08 (±0.004)	0.001 (±0.0002)
pNP-acetate	0.02 (±0.0004)	0.09 (±0.001)	0.002 (±0.0001)

[a] "–" = Activity not detected.

Hydrolysis of Bergamot Peel. To investigate the enzymatic release of pectic oligosaccharides and flavonoids, bergamot peel (1 g) was incubated with 10 U PGase-equivalent activity of Pectinase 690L and 62L or 10 U of cellulase-equivalent activity of C013P in 50 mM Na-acetate buffer (pH 5.0) in a final volume of 100 mL for 24 h under agitation (37 °C or 50 °C, 100 rpm). A combination of Pectinase 62L and Cellulase C013P was also used. Samples were then filtered through GF-C paper under vacuum (Millipore 1225 sampling manifold), and residues were washed with distilled water and were dried (60 °C) to a constant weight before weighing. Both the filtrates and the residues were analyzed for sugar and flavonoid composition.

To evaluate the time release of pectic oligosaccharides and flavonoids, bergamot peel (1 g) was incubated with 10 U PGase-equivalent activity of Pectinase 62L over 24 h in a final volume of 100 mL. Aliquots (5 mL) were removed at defined time points, reactions were terminated by heating to 100 °C for 5 min and were centrifuged (10000*g*, 5 min), and the supernatant was analyzed for flavonoid and sugar solubilization. The amount of flavonoids (glycosides or aglycones) and monosaccharide material released was quantified against standard curves. All assays were prepared and analyzed in duplicate.

HPLC Analysis of Flavonoid Glycosides and Aglycones. The flavonoid glycosides and aglycones released after the various treatments were analyzed using a previously described method (*2*). Essentially, the samples were analyzed using an Agilent binary pump HP1100 HPLC with diode-array detection (270-nm specific wavelength; 200−600 nm overall) for the majority of samples in combination with a Phenomenex Luna C_{18} (*2*) column (250 × 4.6 mm, 5 μm) with a Securityguard precolumn. Selected samples were also analyzed with this HPLC system in combination with an Agilent SL MSD, positive ion mode electrospray mass spectrometry for selected samples. MS was done for additional confirmation of flavonoid glycosides and

Table 2. α-Rhamnosidase and β-Glucosidase Activities in Pectinase 62L and Pectinase 690L Using Different Flavanone Glycosides and One Flavanol Glycoside as Substrates[a]

	Pectinase 62L			Pectinase 690L		
compound	substrate	glucoside	aglycone	substrate	glucoside	aglycone
naringenin 7-*O*-glucoside	33	–	67	0	–	100
	34		66	0		100
naringin (naringenin 7-*O*-neohesperoside)	0	39	61	30	0	70
	0	38	62	31	0	69
eriodictyol 7-*O*-glucoside	32	–	68	0	–	100
	34		66	0		100
neoeriocitrin (eriodictyol 7-*O*-neohesperoside)	8	0	92	98	0	2
	7	0	93	92	0	8
hesperidin (hesperetin 7-*O*-rutinoside)	0	52	48	77	0	23
	0	53	47	78	0	22
neohesperidin (hesperetin 7-*O*-neohesperoside)	2	65	33	67	2	31
	2	66	32	66	0	34
rutin (quercetin 3-*O*-rutinoside)	53	35	12	90	6	4
	53	36	11	90	7	3

[a] Data expressed as percentage form of total μmoles in duplicate assays, i.e., initial substrate, glucoside (for rhamnoglucosides), and aglycones. –, not applicable.

aglycones in the samples. Calibration curves for the major flavonoid glycosides and their corresponding aglycones were prepared on the basis of the previous evaluation of bergamot peel flavonoids (*2*).

Gas Chromatographic Analysis of Sugars. Sugars were released from the samples after hydrolysis with 72% H_2SO_4 for 3 h followed by dilution to 1 M (Saeman hydrolysis). Hydrolyzed monosaccharides were analyzed as their alditol acetates by gas chromatography (GC) (*15*). Total uronic acid content was determined colorimetrically at 580 nm using a previously reported method (*16*), and values were compared to a standard curve of galacturonic acid (0−40 μg/mL).

Size-Exclusion Chromatography of Mono- and Oligosaccharides. The fractionation system was comprised of a triplet of TSK-GEL stainless steel columns (TosoHaas, Montgomeryville, PA) arranged in series using a previously described method (*17*). The first column was a G3000PW with a molecular weight range of up to 5×10^4, and the second was a G4000PW with a range of 2×10^3 to 3×10^5. Finally, the third column was a G6000PW, possessing a range of 4×10^4 to 8×10^6. The mobile phase (50 mM Na-acetate buffer, pH 5.0) filtered with a 2-μm sintered filter at source and a protective 2-μm "frit" filter was situated between the pump outlet and the column inlet. Samples were loaded onto the column at a flow rate of 0.5 mL/min, and peaks were monitored by a Gilson 132 RI detector (Anachem, United Kingdom) operated in analytical mode, with a cell volume of 8 μL per channel. Data was recorded and analyzed using Hewlett-Packard Chemstation. Column calibration was performed with a variety of standards, principally pullulans (Polymer Labs, Church Stretton, Shropshire, United Kingdom). A typical run time for each sample was 90 min.

RESULTS

Enzyme Activities (Pectinase 62L, Pectinase 690L, Cellulase C013P). The activities detected in the pectolytic and cellulolytic enzyme preparations used in this study are shown in **Table 1**. No activity against orange peel polygalacturonic acid, sugar beet arabinan, pNP-α-rhamnopyranoside, and pNP-α-glucopyranoside was detected in the Cellulase CO13P preparation, while no activity against carboxymethyl cellulase was detected in Pectinase 62L.

Incubation of individual flavanone glycosides and flavonol glycoside showed that Pectinase 62L and 690L contained both α-rhamnosidase and β-glucosidase activities capable of removing the sugars attached to the substrates (**Table 2**). The enzyme preparations do not contain direct "rutinase" activity, that is, activity against the rhamnose−glucose disaccharide. Pectinase 62L had greater rhamnosidase than glucosidase activities, whereas Pectinase 690L had a higher glucosidase than rhamnosidase activity. The cleavage activities of both Pectinase 62L and 690L were much higher with flavanone glycoside substrates than with the flavonol glycoside (rutin). No flavanone rhamnosides are commercially available, and therefore rhamnosidase activity alone against these substrates was not determined. Therefore, the combination of rhamnosidases and glucosidases cleaves the Glc-Rha disaccharide from the flavonoids in two steps (i.e., rhamnose followed by glucose) rather than a single rutinase-like activity.

Flavonoids Released over 24-h Incubation. Analysis of the flavonoids released from the bergamot peel after treatment with the three food-grade enzymes (**Figure 1A−C**) and a combination of Pectinase 62L with Cellulase C013P (**Figure 1D**) for 24 h showed different patterns. Essentially, treatments of the peel with Pectinase 62L, Pectinase 690L, or Pectinase 62L/Cellulase C013P led to the release of approximately 50% of the total flavonoids as their aglycones in the soluble fraction (compared with the theoretical 100% release from 1 g of bergamot peel) (**Figure 2**). Treatment with Cellulase C013P alone had no effect on glycoside release above that found with nonenzyme-treated controls, and no hydrolysis of flavonoid glycosides to their corresponding aglycones occurred. Buffer-only control samples had the same profile as the Cellulase-treated samples, that is, simply solubilization of the glycosides during the incubation. There was no hydrolysis of the glycosides in the buffer-only control samples (data not shown). LC-MS analysis of the peel residue after Pectinase 62L, Pectinase 690L, and Pectinase 62L/Cellulase C013P treatments showed that the aglycones were also present in the residues. Combined yields of all aglycones in soluble plus residue (with the exception of hesperetin) were 80−90% compared with the calculated 100% (**Figure 2**). In the peel residue, after all four treatments, the concentrations of bergamottin and bergapten were higher than in the soluble fractions, again indicative of their poor aqueous solubility.

In addition, liquid chromatography coupled with mass spectrometry (LC-MS) of the supernatant post-peel treatment with Pectinase 62L, 690L, or the combined Pectinase 62L and Cellulase C013P showed the formation of a novel flavanone glycoside; it had a UV−vis spectra that was identical to other flavanones, and it had a core aglycone of hesperetin ([aglycone + $H]^+ = 303$) and an MW of 608 ([M + $H]^+$ molecular ion = 609) compared with neohesperidin ([aglycone + $H]^+ = 303$, [M + $H]^+$ molecular ion = 611). This suggests that a dehydrogenation reaction may have occurred involving the loss of two hydrogen atoms from one of the sugars of neohesperidin

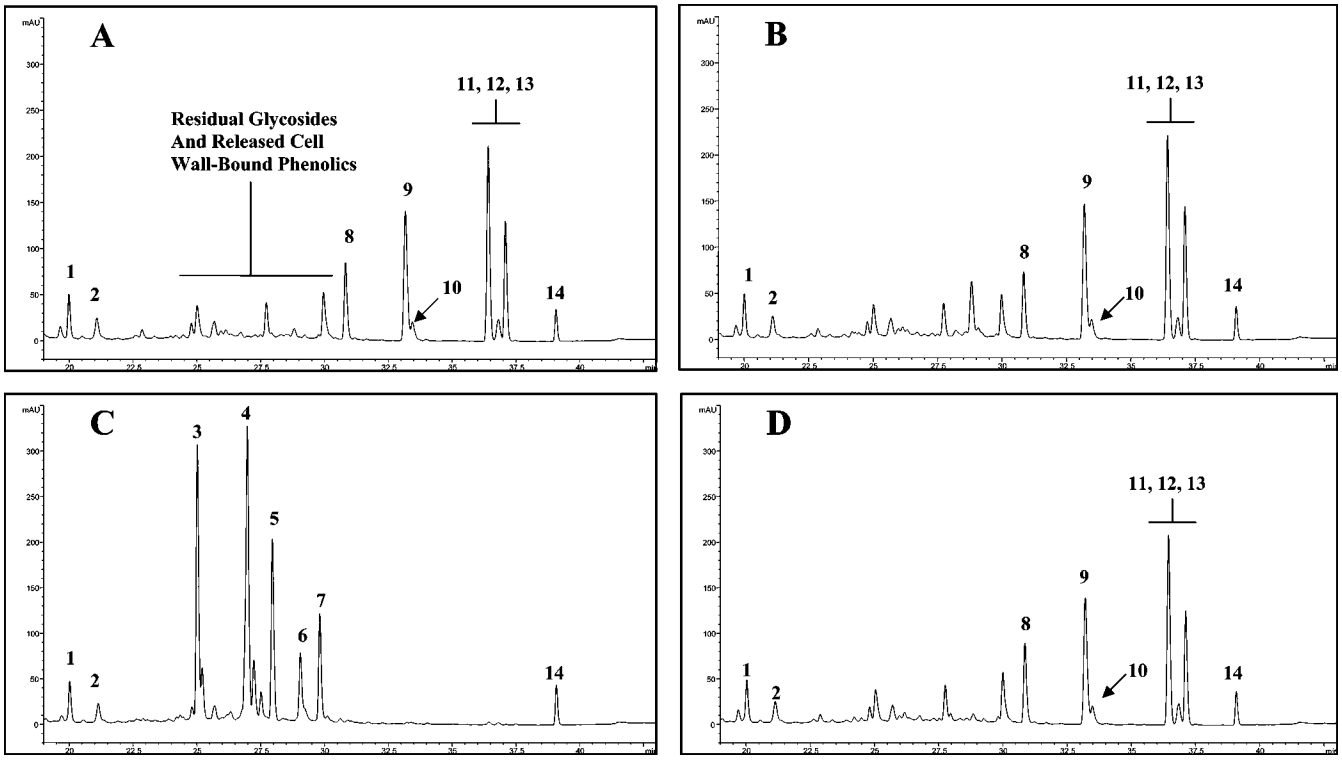

Figure 1. Flavonoid glycosides and aglycones in soluble fractions after various enzyme treatments. Chromatograms at 270 nm after treatment with (**A**) Pectinase 62L, (**B**) Pectinase 690L, (**C**) Cellulase C013P, and (**D**) a mixture of Pectinase 62L and Cellulase C013P. Only the major peaks are labeled; for details, see **Table 2** and ref *2*. Peak ID: 1 = apigenin 6,8-di-*C*-glucoside, 2 = diosmetin 6,8-di-*C*-glucoside, 3 = neoeriocitrin, 4 = naringin, 5 = neohesperidin, 6 = naringenin monorhamnoside, 7 = hesperetin monorhamnoside, 8 = dehydo-neohesperidin, 9 = eriodictyol (aglycone), 10 = apigenin (aglycone), 11= naringenin (aglycone), 12 = diosmetin (aglycone), 13 = hesperetin (aglycone), 14 = bergapten (a psoralen, i.e., a furanocoumarin).

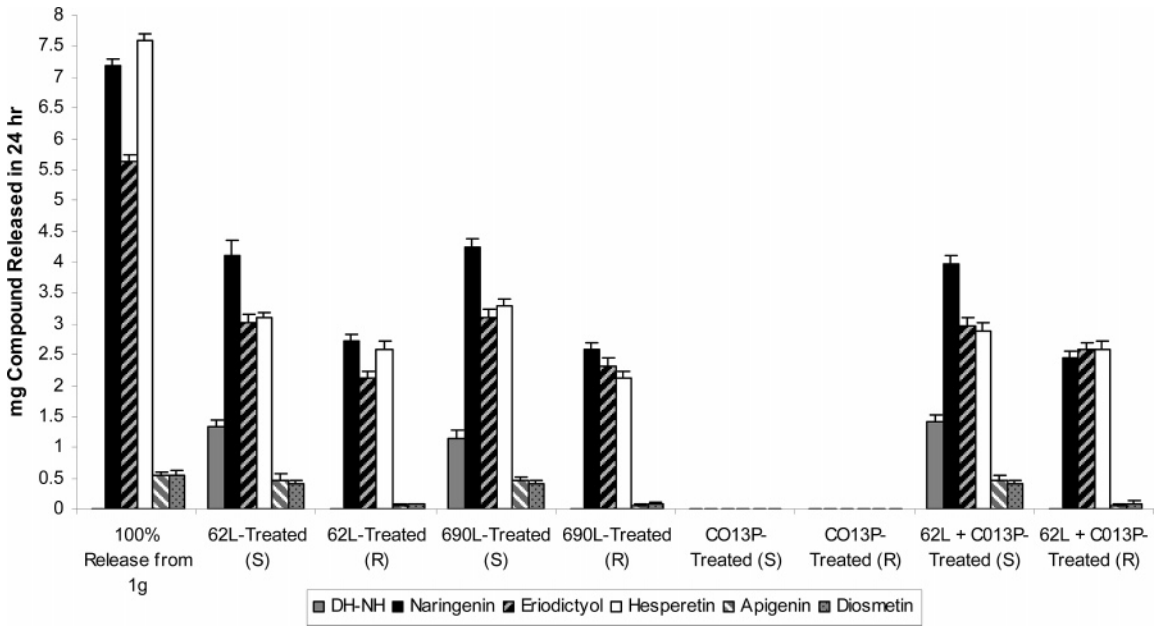

Figure 2. Dehydro-neohesperidin (DH-NH) and aglycones in solubilized (S) and residual (R) material after treatment of bergamot peel with Pectinase 62L, Pectinase 690L, Cellulase C013P, and a combination of Pectinase 62L + Cellulase C013P. No dehydro-neohesperidin was detected in the peel residue. The flavone aglycones (apigenin and diosmetin) were only present in low amounts in the residue.

(the major bergamot peel hesperetin glycoside). This reaction did not occur with any of the other glycosides in the peel nor did it occur when the pure substrates, including neohesperidin, were incubated with Pectinase 62L or 690L. This suggests that possibly cell-wall enzymes in the peel, not present in the Pectinase 62L or 690L, may have been released and may have catalyzed the dehydrogenation of neohesperidin. Apigenin di-*C*-glucoside and diosmetin di-*C*-glucoside were unaffected by any of the enzyme treatments, and their concentrations were constant irrespective of treatment.

Sugars Released over 24-h Incubation. The residues after 24-h incubations were analyzed to determine the composition of the polysaccharides remaining (**Table 3**). The main sugar present in the residue was glucose, with 29%, 70%, 49%, and 35% of the original level still remaining after treatment with Pectinase 690L, Pectinase 62L, Cellulase C013P, and Pectinase

Table 3. Sugar Composition of Bergamot Starting Material and Residues after Treatment with Pectinase 690L, Pectinase 62L, Cellulase C013P, and Pectinase 62L + Cellulase C013P[a]

sample	biomass solubilization (%)	residual sugars				
		Ara	Xyl	Gal	Glc	GalA
bergamot peel (control)	0	64 ± 0.2	16 ± 0.7	48 ± 0.1	245 ± 2.2	256 ± 9. 6
residue post-pectinase 690L	82	19 ± 0.2	37 ± 1.1	29 ± 1.3	284 ± 15.6	79 ± 4.8
residue post-Pectinase 62L	70	15 ± 0.9	33 ± 1.3	29 ± 1.6	403 ± 12.9	71 ± 4.4
residue post-Cellulase C013P	62	63 ± 2.3	26 ± 1.5	48 ± 0.9	224 ± 4.9	214 ± 5.5
residue post-Pectinase 62L + Cellulase C013P	81	21 ± 0.8	35 ± 0.9	29 ± 1.0	259 ± 7.6	63 ± 1.8

[a] Values are expressed as μg compound mg^{-1} material. GalA represents galacturonic acid.

62L + Cellulase C013P, respectively. The highest percentage of biomass reduction was achieved by using Pectinase 690L (96% of the solubilized material was sugar), followed by Pectinase 62L (68%) and Cellulase C013P (73%). While the combination of Pectinase 62L + Cellulase C013P resulted in the same degree of solubilization (81%) as Pectinase 690L, the amount of sugars released was lower (83%). These results do not significantly differ from those on AIR residue posttreatment with the same glycoside hydrolase preparations previously reported (*2*). Bergamot alcohol-insoluble residue (AIR) is a flavonoids-free extract, thus indicating that flavonoids in the peel do not have an effect on enzyme activities in terms of sugar solubilization. The main sugars solubilized over the 24-h hydrolysis were glucose and galacturonic acid (results not shown), showing the preferential enzyme-catalyzed solubilization of pectin and cellulose from bergamot peel by these enzymes. Smaller amounts of arabinose were solubilized over the 24-h incubation.

Time-Course Release of Sugars and Flavonoids. A time-course study on the release of flavonoids from bergamot peel using Pectinase 62L showed that conversion of glycosides into aglycones was gradual over an 8-h period, followed by a more rapid conversion rate up to 24 h (**Figures 3** and **4A**). In control samples, there was approximately a 2-fold increase in flavonoid glycosides from $T = 0$ (initial addition of the enzyme-free buffer to 1 g peel) to $T = 24$ h at 37 °C, representing approximately 75−80% of the potential total glycosides in 1 g of peel (data not shown). After 24-h incubation, only residual intact peel glycosides remained in the soluble fraction. Apigenin di-*C*-glucoside and diosmetin di-*C*-glucoside were unaffected by the enzymatic treatment. In addition, several minor simple phenolic acids (benzoic and hydroxycinnamic) were detected in the 24-h samples, further indicating the extensive solubilization of the cell wall material through hydrolysis of phenolic-sugar linkages. The main sugars solubilized over the 24-h hydrolysis were rhamnose, galacturonic acid, and glucose (**Figure 4B**). Over 80% of the total rhamnose in the peel was released within 24-h hydrolysis. Rhamnose can arise from both pectin (rhamnogalacturonan-type) and the flavonoids. Seventy percent of the uronic acid was released. Only a small amount of glucose (28%) was solubilized, coming mainly from residual flavonoids and β-glucan in the peel rather than major cellulose degradation. From size-exclusion chromatography of the solubilized material, initially oligomers of approximately eight sugars were produced as a result of the 2-h enzymatic hydrolysis, which were subsequently broken down into lower molecular weight carbohydrates, predominantly monosaccharides, by the 24th hour of incubation because of the presence of glycosidases in the Pectinase 62L preparation.

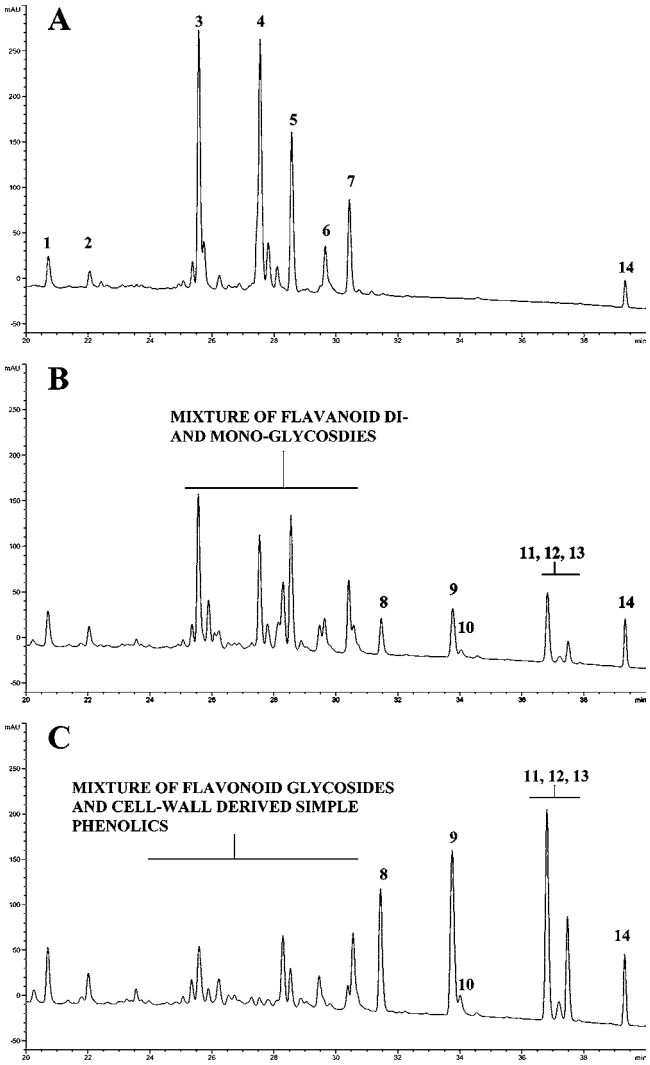

Figure 3. Example chromatograms (270 nm) of soluble fraction from Pectinase 62L time-course study. $T = 0$ min (**A**), 8-h postincubation (**B**), and 24-h postincubation (**C**). Only the major peaks are labeled; for details of the minor flavonoids, see ref *2*. Peak ID: 1 = apigenin 6,8-di-*C*-glucoside, 2 = diosmetin 6,8-di-*C*-glucoside, 3 = neoeriocitrin, 4 = naringin, 5 = neohesperidin, 6 = naringenin monorhamnoside, 7 = hesperetin monorhamnoside, 8 = dehydo-neohesperidin, 9 = eriodictyol (aglycone), 10 = apigenin (aglycone), 11= naringenin (aglycone), 12 = diosmetin (aglycone), 13 = hesperetin (aglycone), 14 = bergapten (a psoralen, i.e., a furanocoumarin). Some additional minor peaks appearing after enzyme incubation were identified by LC-MS as monoglucosides of the various flavanones derived from hydrolysis of the major rutinosides.

DISCUSSION

The pectinase and cellulase enzymes used in this study contain a balance of main-chain and side-chain-acting enzymes (**Table 1**) capable of significantly reducing the biomass of bergamot peel, a byproduct of the essential oil manufacturing industry, producing low molecular weight pectic oligosaccharides and a

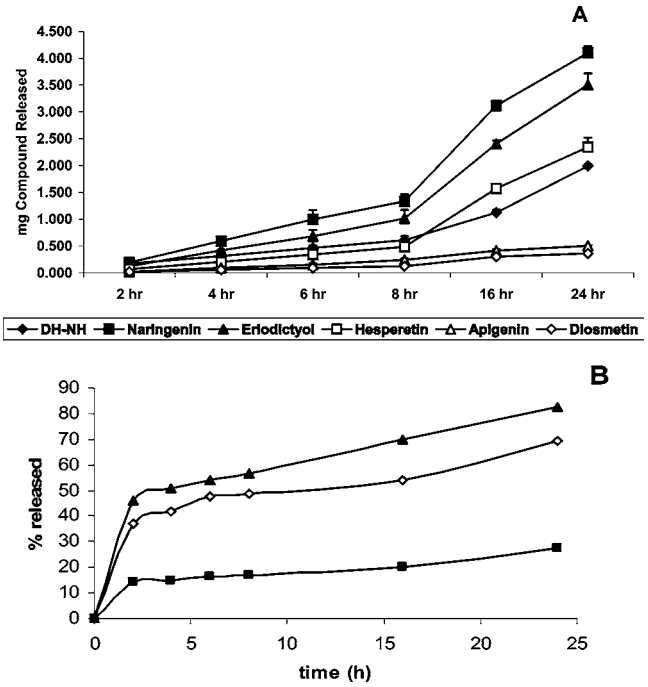

Figure 4. Time-course release curves of aglycones and dehydroneohesperidin (**A**) and sugars (**B**) in the soluble fraction after treatment of bergamot peel with Pectinase 62L. Maximal release of aglycones at 24 h represents the following percentages of each aglycone from 1-g peel (theoretical 100%): naringenin (57%), eriodictyol (61.7%), hesperetin (30.7%), apigenin (100%), and diosmetin (100%). The major sugar released was rhamnose (▲) followed by galacturonic acid (◇) and glucose (■).

mixture of glycosylated and deglycosylated flavonoids. As the composition of bergamot peel is similar to that of other *Citrus* fruit peels, these enzymes could be employed in the bioconversion of these byproducts throughout the *Citrus*-processing industry. Pectinase and cellulase combinations have previously been used to liquefy apple pomace, a byproduct from the apple processing industries (*18*−*20*), where enzyme treatment of the mash reduces the water-binding capacity of the cell wall pectins.

Pectin is a component of dietary fiber, and incorporating fiber into frequently consumed foods would help to overcome the current fiber deficit. The albedo of *Citrus* fruits is a rich source of both dietary fiber and bioactive flavonoids. *Citrus* peel extract, whether as polymeric fibers or shorter oligosaccharides, has the potential to be included as a natural, cost-effective antioxidant in meat-based food matrices because of both the polysaccharide and the polyphenol content (*20*, *21*). Pectins are extensively degraded in the colon (*22*) where they may exert a prebiotic-type effect (*23*, *24*). The fermentability of the pectins are dependent on the particle size of the cell-wall-derived material that contains them (*25*), and treatment with enzymes, such as Pectinase 62L and 690L, can act as an aid to break down these components to release prebiotic-type material from bergamot peel.

The results shown in **Figure 2** demonstrate that all the flavonoid deglycosylation activity is coming from the Pectinase 62L preparation, which is surprising, as it would be expected that the β-glucosidase present in Cellulase CO13P would make a contribution. This suggests that the *Aspergillus* sp. β-glucosidase works better on bergamot flavonoid deglycosylation in comparison with similar activities from the *Trichoderma* sp. producing the Cellulase CO13P components. While rutinase activity was first reported in fungal enzyme preparations recently (*26*), the enzyme mixtures used in this study did not contain such activity. The deglycosylation of the rutinosides present in bergamot peel must therefore be catalyzed by the synergistic interactions between the α-rhamnosidase and the β-glucosidase in the pectinase components of both Pectinase 62L and 690L.

Citrus polyphenols, flavonoids, and polymethoxyflavones (in orange peel) are potent natural antioxidants, and the catechol group plays an important role in their functionality (*27*). The identification of deglycosylating activities, especially α-rhamnosidase activity, in these enzyme preparations has important implications for the potential use of bergamot peel-derived flavonoids as health-promoting compounds. Many of the ingested dietary flavonoids (e.g., rutin, rutinosides, neohesperosides, and complex acylated flavonoid glycosides) reach the colon without degradation. In the colon, they are substrates for the complex indigenous microflora which are known to contain species and genera able to exert varying effects on the health of the host (*28*−*30*). While the majority of dietary phytochemicals are hydrolyzed by the colonic microflora, small percentages are taken up during transit through the small intestine, and in some cases, the presence of a glucose moiety may enhance absorption (*31*, *32*). It has also been demonstrated that the bioavailability of some xenobiotics is dependent mainly on small intestinal uptake (*33*, *34*). Polyphenol glycosides, however, are relatively hydrophilic and do not diffuse passively across biological membranes. Simple flavonoid glucosides can be taken up into cells via SGLT1 (sodium-dependent glucose transporter 1) and other hexose transporters, and the aglycones are readily absorbed by passive diffusion. Rutinosides and some other glycosides are not absorbed in the small intestine. Thus, a deglycosylation step is critical for the absorption of dietary flavonoids (*35*). The low solubility of the released flavonoid aglycones could be increased by addition of food/pharmaceutical grade cyclodextrins as has been recently demonstrated for naringenin and hesperetin (*36*, *37*).

In conclusion, the enzyme preparations employed in this study, Pectinase 62L, Pectinase 690L, and Cellulase CO13P, could be used to develop new processes for the utilization of *Citrus* and other pectinaceous byproducts. In this case, solid material has been efficiently processed, as compared with previous studies of juices (*3*, *4*). However, their use should be tailored to the desirable end product, because treatment of fruit juices with fungal pectinases (Viscozyme) were shown to markedly reduce the antioxidant activity of the whole extract (*26*).

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