JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Association between Polymerization Degree of Apple Peel Polyphenols and Inhibition of *Helicobacter pylori* Urease

EDGAR PASTENE,*,† MIRIAM TRONCOSO,‡ GUILLERMO FIGUEROA,‡ JULIO ALARCÓN,§ AND HERNÁN SPEISKY†

Laboratory of Antioxidants, Institute of Nutrition and Food Technology, University of Chile, Av. Macul 5540, Santiago 138-11, Chile, Laboratory of Microbiology, Institute of Nutrition and Food Technology, University of Chile, Av. Macul 5540, Santiago 138-11, Chile, and Laboratory of Synthesis and Natural Products, Department of Basic Sciences, Faculty of Sciences, University of Bío-Bío, Avenida Andrés Bello, s/n P.O. Box 447, Chillán, Chile

Apple peel extracts and their fractions pooled according to their molecular size were prepared and evaluated for their inhibitory activity against *Helicobacter pylori* and Jack bean ureases. Urease Inhibitory effect of apple peel polyphenols (APPE) extracted from the Granny Smith variety was concentration-dependent and reversible. High molecular weight polyphenols (HMW) were more active against *Helicobacter pylori* and Jack bean ureases than low molecular weight polyphenols with IC_{50} values of 119 and 800 μg GAE/mL, respectively. The results suggest that monomeric compounds (mainly flavan-3-ols-and quercetin-*O*-glycosides) will not be implicated in the antiurease effect displayed by the apple peel polyphenolic extract. Thus, as a byproduct, apple peel is suitable for developing functional ingredients that could be useful for neutralizing an important *Helicobacter pylori* colonization factor.

KEYWORDS: ***Helicobacter pylori*; urease; apple; *Malus domestica*; polyphenols; procyanidins**

INTRODUCTION

Health benefits of apple polyphenols have been widely investigated during the past decade (*1*). Apples are a rich source of polyphenols, which are distributed in the pulp, seeds, and peel (*2*, *3*). The *in vivo* protective effects of apple cloudy juices have been demonstrated in rats treated with the colon carcinogenic agent 1,2-dimethylhydrazine, DMH (*4*). Recently, an inhibitory effect of apple polyphenols on growth was observed on adenoma (HT29) and colon carcinoma (LT97) cells lines (*5*). At a molecular level, the inhibition of HT29 cell proliferation induced by the apple polyphenols imply both an inhibition of epithelial grow factor receptor (EGFR) autophosphorylation and an activation of caspases (*6*, *7*). In addition, investigations on the epigenetic effects of apple polyphenols on cell growth, conducted in colon cancer cells, have demonstrated an inhibition of the expression of enzymes involved in gene methylation (DNA methyl transferases) and a reactivation of tumor suppressor genes (*8*).

Among several varieties, Granny Smith apples are exported both as fresh product and as concentrate for apple juice production. Although a great part of such exports consider the whole fruit, during the past decade, exports of dehydrated apple products have had a significant increase. Since apple dehydration requires peel removal, important amounts of apple peel are produced and classified as agro-industrial waste. Previous studies indicated that some apple varieties could have from 40 to 50% of the total fruit polyphenols in the peel (*9*). In fact, the concentration of polyphenols in peel could be up to three times higher than that found in pulp. The principal classes of whole apple polyphenols include flavonoid glycosides, phenol carboxylic acids esters, dihydrochalcones, catechins, and procyanidins (*10*). The latter and quercetin glycosides represent up to 60% and 18% of the apple peel polyphenols, respectively (*11*).

Recently, various studies evaluated a possible gastrointestinal protective role of apple polyphenols. For instance, an antiulcerative effect of apple polyphenol extract was reported previously in rats given the extract during ten days before inducing gastric injury with indomethacin (*12*). In the same study, it was observed in vitro, that the oxidative damage induced by either indomethacin and/or by xanthine-xanthine oxidase (X/XO) in MKN 28 cells (a gastric epithelium cell line) can be ameliorated by the prior addition of the apple polyphenols to the culture. More recently, the same group demonstrated a protective effect of the apple polyphenol extract in rats in which gastric damage was induced by aspirin (*13*). As expected, differences in the mechanism and magnitude of the protection induced by apple polyphenols may emerge using different injury models, apple varieties, and extract preparations. For instance, when compared

† Laboratory of Antioxidants, University of Chile.
‡ Laboratory of Microbiology, University of Chile.
§ University of Bío-Bío.

10.1021/jf8025698 CCC: $40.75
Published on Web 01/07/2009

Table 1. Phenolics Composition of Fresh Apple Peel and APPE Extract Obtained from Granny Smith Apples

compound	fresh apple phenolics (%)[a]	APPE phenolics (%)[a]
	flavonoids	
rutin	3.57 ± 0.50	3.80 ± 0.76
hyperoside	21.08 ± 1.20	19.70 ± 2.20
isoquercitrin	6.25 ± 2.00	8.80 ± 1.81
quercetin-3-*O*-pentosides[b]	18.01 ± 1.54	17.40 ± 3.54
quercitrin	9.01 ± 1.88	7.80 ± 2.01
quercetin	0.09 ± 0.03	0.14 ± 0.08
∑ flavonoids	**58.01 ± 1.19**	**57.64 ± 1.73**
	flavan-3-ol monomers	
epicatechin	6.20 ± 0.10	5.60 ± 1.89
catechin	Nd[d]	0.05 ± 0.01
∑ flavan-3-ol monomers	**6.20 ± 0.10**	**5.65 ± 0.95**
∑ total procyanidins[c]	**22.09 ± 3.00**	**24.38 ± 2.78**
	dihydrochalcones	
phloridzin	13.05 ± 3.22	11.34 ± 4.12
phloretin-2′-xyloglucoside	0.10 ± 0.05	0.14 ± 0.06
∑ dihydrochalcones	**13.15 ± 1.64**	**11.48 ± 2.09**
	phenol carboxylic acids	
chlorogenic acid	0.55 ± 0.01	0.80 ± 0.06
caffeic acid	Nd	0.05 ± 0.01
∑ carboxylic acids	**0.55 ± 0.01**	**0.85 ± 0.04**
total	**100 ± 1.19**	**100 ± 1.52**

[a] Data are the average of triplicates determined by the HPLC method and expressed as the percentage of the total polyphenolic content measured by the Folin−Ciocalteu method. [b] Calculated on the basis of rutin measured from RP-HPLC. [c] Estimated by the RP-HPLC method after phloroglucinolsis. [d] Nd = not detected.

by their antiulcerative properties, Chinese quince- and apple-polyphenols showed differences on an HCl/ethanol-induced ulcer model (*14*). Although both extracts were found to be dose-dependently effective, at the highest dose, the apple extract preparation showed to be pro-ulcerative rather than gastro-protective. According to the authors, the deleterious action could be ascribed to the comparatively higher presence of chlorogenic acid found in the apple preparation. Previously, using the same injury model, chlorogenic acid had been reported to be pro-ulcerogenic in rats (*15*). Interestingly, in varieties such as Granny Smith, the content of chlorogenic acid in apple peel is considerably lower than that in pulp (*16*).

In addition to its gastro-protective effects on chemically induced injury, apple polyphenols may also contribute to ameliorate chronic gastro-intestinal affections induced by *Helicobacter pylori*. The latter is a Gram-negative spiral bacterium that infects about 50% of the world's population; it is the only microorganism known to permanently inhabit the human stomach (*17*). Many studies have established *H. pylori* as an etiologic agent for gastric cancer, mucosa-associated lymphoid tissue (MALT) lymphoma, and peptic ulcer (*18, 19*). In terms of its capacity to colonize the gastric mucosa, one of the most important features of *H. pylori* is its extremely high capacity to produce urease, whose main function is buffering *H. pylori*'s periplasm (*20*). Also, urease-generated ammonia neutralizes gastric acidity and thereby promotes within, the gastric lumen, a neutral microenvironment surrounding the bacterium. Considering the crucial role urease plays in *H. pylori* survival and gastric colonization, we undertook a study to assess the effect of a polyphenol-rich extract, obtained from Granny Smith apple peel wastes. In view of its richness in procyanidins, we investigated the existence of a relationship between the degree of polymerization of the procyanidins contained in such an extract and their possible inhibitory effect on *H. pylori* urease.

MATERIAL AND METHODS

Standards, Chemicals, and Solvents. Gallic acid, chlorogenic acid, caffeic acid, (+)-catechin, (−)-epicatechin, phoridzin, quercetin, quercetin 3-*O*-rutinoside, procyanidin B1 and B2, toluene-α-thiol, cysteamine, phloroglucinol, sodium carbonate, and the Folin−Ciocalteu were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavonol glycosides (quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside and quercetin 3-*O*-rhamnoside were from Roth (Karlsruhe, Germany). Procyanidin C1 was purified according to Sun and co-workers (*21*). All other solvents were HPLC grade purchased from Merck (Darmstadt, Germany).

***Helicobacter pylori* Strains and Culture Conditions.** *Helicobacter pylori* (ATCC 43504) was kindly provided by Professor Apolinaria García (Universidad de Concepción, Chile). Strains were incubated for 4 days in a microareobic gas environment (15% CO_2, 5% O_2, and 80% N_2) in Brucella broth containing 5% horse serum and 0,1% β-cyclodextrin.

***H. pylori* Urease.** Urease extraction was carried out according to the protocol suggested by Xiao and co-workers (*22*), with minor modifications. Briefly, 100 mL broth cultures (optical density at 600 nm of 0.25 corresponding to 1×10^8 CFU/mL) were centrifuged (5000*g*, 30 min, 4 °C) to collect the bacteria, and after washing twice with phosphate-buffered saline (pH 7.7), the *H. pylori* pellet was stored at − 70 °C. The *H. pylori* pellet was returned to room temperature, and after the addition of 3 mL of distilled water and protease inhibitors, sonication was performed for 60 s. Following centrifugation (14,000*g*, 15 min, 4 °C), the supernatant was desalted through a Sephadex G-25 column (Sigma). The eluted crude urease was further concentrated 5-fold using a centrifugal filter device 50,000 Da NMWL (Millipore Corporation, Bedford, MA) at 4 °C. This solution was added to an equal volume of glycerol and stored at −20 °C until use. Total protein was evaluated by the Bradford method (Sigma) with bovine serum albumin as the standard. Urease activity was assessed by measuring ammonia production using the indophenol method as described by Weatherburn (*23*). One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea (producing 2 μmol of ammonia) per min per mg of total protein. The amount of ammonia released was determined from a standard curve.

Preparation of Apple Peel Extracts and Fractionation. Apple peels from ripe fruits of the cultivar Granny Smith (*Malus domestica* cv. Granny Smith) were kindly provided by SURFRUT Ltda. (Santiago, Chile). Briefly, frozen peels (−20 °C) were mixed with hot water (9 + 1 w/v; 5 min, 90 °C) and then pressed through a stainless steel grid (15 μm). The cake was treated with water at 65 °C using an Ultraturrax homogenization device (1 min). Homogenized peels were macerated during 30 min and filtered through the stainless steel grid. Pooled aqueous cloudy extracts were retained on absorber resin Sepabeads SP-850 (Supelco, Bellefonte, USA) that was packed in a glass column (50 mm i.d. × 300 mm). Water soluble ingredients such as sugars, organic acids, and minerals were removed by washing first with five volumes of distilled water. Retained compounds were then eluted with 70% ethanol. The ethanolic fraction was concentrated by evaporative rotation (<40 °C) and dried under vacuum in a dessecator. Dried apple peel polyphenol-rich extract (APPE) was stored at − 70 °C until use.

For HPLC analysis of fresh apple peel, ∼10 g of frozen peel was weighed and then transferred to a beaker with 70% chilled aqueous acetone, considering a 1 + 9 w/v ratio for the extraction of total polyphenols including polymeric procyanidins. The mixtures were homogenized using Ultraturrax and filtered first through a Whatman no.1 filter paper and then through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA). The final filtrate was injected directly onto a liquid chromatograph.

To obtain low molecular weight polyphenols, APPE was suspended in water and extracted with ethyl acetate (5 times). The ethyl acetate extracts (EAE) were pooled and concentrated under vacuum. The aqueous layer (AQUO) was saved. Dried EAE was further chromatographed on a Toyopearl HW-40s size-exclusion chromatography column (30 × 2.5 cm, Tosoh, Tokyo) with a linear gradient of 5% to 100% of methanol and then to 60% acetone for elution of retained polymeric material. Nine fractions were collected. Fractions I−VI containing low molecular weight compounds were concentrated and dried under

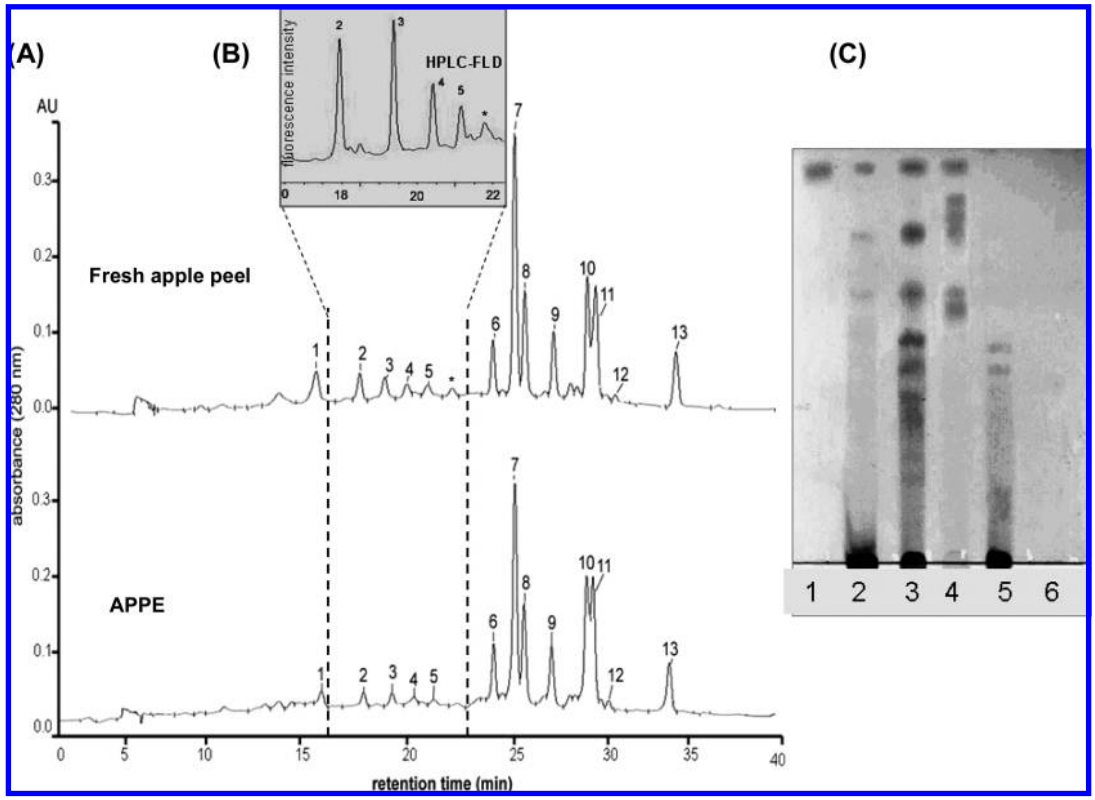

Figure 1. Reverse-phase HPLC-DAD profiles of APPE and fresh apple peel: (**A**) Comparison between HPLC profiles of fresh apple peel and APPE recorded at 280 nm showing the peaks of (1) chlorogenic acid, (2) procyanidin B1, (3) procyanidin B2, (4) (−)-epicatechin, (5) procyanidin C1, (*) unknown, (6) rutin, (7) hyperoside, (8) isoquercitrin, (9) quercetin-3-*O*-pentoside, (10) quercetin-3-*O*-pentoside, (11) quercitrin, (12) phloretin-*O*-xyloglucosid, and (13) phloridzin. (**B**) Insert: close-up of the APPE flavan-3-ols and procyanidins region as detected by HPLC-FLD with excitation at 280 nm and emission at 310 nm. Key for polyphenols is the same as that for the HPLC-DAD trace. (**C**) Chromatographic separation by TLC of the samples was visualized with DMACA reagent. Lane 1, (−)-epicatechin; lane 2, fresh apple peel extracts; lane 3, APPE; lane 4, LMW; lane 5, HMW; and lane 6, rutin.

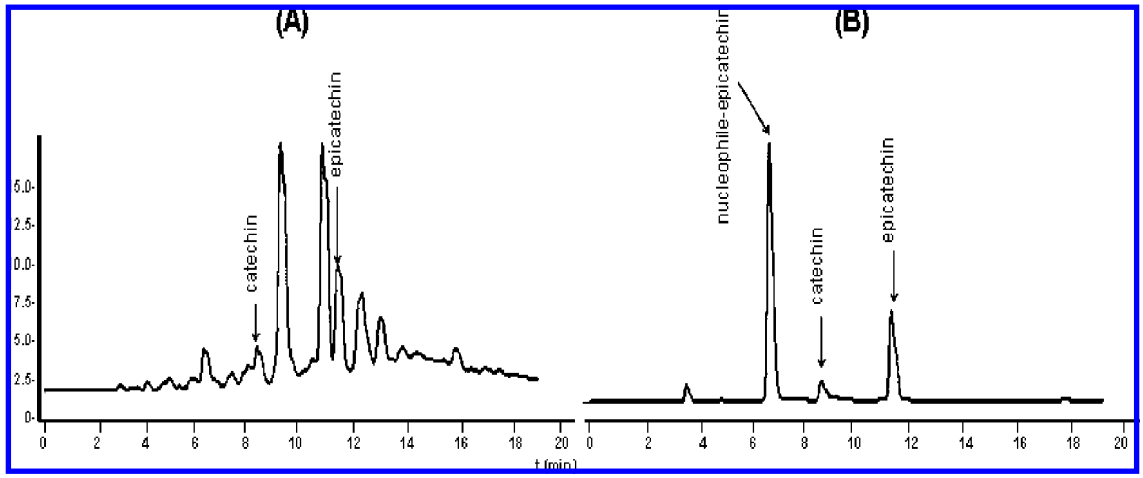

Figure 2. Depolymerization of APPE. RP-HPLC of APPE before (**A**) and after (**B**) degradation with phloroglucinol.

vacuum (LMW). The AQUO fraction was loaded on a Sephadex LH-20 column (30 mm i.d. × 300 mm) activated previously with water during 24 h. The column was eluted with methanol containing decreasing proportions of water and methanol alone, and finally, the polymeric material was recovered with 60% acetone, concentrated, and dried under vacuum (high molecular weight compounds, HMW). TLC was carried out on 20 × 20 cm silica gel 60 F_{254} plates (Merck), eluted with toluene−acetone−formic acid (3:6:1) (*24*).

Flavan-3-ols and procyanidins were detected by staining with dimethylaminocinnamaldehyde (DMACA). Total polyphenolic contents (TPC) were determinate with Folin−Ciocalteau reagent and expressed as mg of gallic acid equivalents (GAE) per gram of dried extract. The equation of gallic acid calibration curve was $y = 0.091x + 0.0229$ ($r^2 = 0.9951$).

High-Performance Liquid Chromatography (HPLC). Extracts and fractions were separated by RP-HPLC using an Agilent 1100 and a Lachrom instrument, both equipped with a 250 × 4.6 mm, 5 μm, Kromasil KR100−5C18 column (Eka Chemicals AB, Bohus, Sweden). The solvent system was composed of solvent A (double distilled water containing 0.1% TFA, v/v) and solvent B (acetonitrile containing 0.1% TFA). The following gradient system was used: 0−25 min, 10−30% B; 25−30 min, 30−75% B; 30−35 min; 75−10% B at a flow of 1

Table 2. Total Polyphenol Content, Mean Degree of Polymerization and Urease Inhibitory Activities of Fresh Apple Peel, APPE, and Fractions Obtained by Size-Exclusion Chromatography Procedures

extract/ fraction	TPC (mg GAE/g dw)[a]	mDP[b]	IC_{50} Jack bean urease[c] (μg/mL)	IC_{50} *H. pylori* urease[c] (μg/mL)
fresh apple peel	3.50 ± 0.8	2.9 ± 0.2	Nd[d]	Nd
APPE	610 ± 12	3.0 ± 0.5	180	516
LMW	480 ± 8	1.0 ± 0.2	594	800
HMW	520 ± 10	9.5 ± 2	103	119

[a] Mean values and ± SD ($n = 3$) expressed in milligrams of gallic acid (GAE) equivalents per gram of dry weight (either peel or powder). [b] Numbers represent the ratio between the moles of phloglucinol-derived flavan-3-ols and the moles of released-monomeric units. [c] Urease inhibitory activity was determined after 30 min of preincubation with the extract and its fractions. [d] Nd: not determined.

mL/min. For flavonoids, detection was at 280 nm using a diode array detector. For procyanidins and catechins, detection was done using a fluorescence detector with excitation at 280 nm and emission detection at 310 nm. Known compounds were identified by matching their retention times (t_R) and online UV spectra with those of reference substances. Although the other compounds (particularly some quercetin-3-*O*-pentosides) could not be wholly identified, they were characterized according to their class on the basis of their UV−vis spectra. Quantification of quercetin glycosides, chalcones, phenol carboxylic acids, epicatechin, catequin, and procianidins B1, B2, and C1 was carried out using peak areas from external calibration curves.

Mean Degree of Polymerization of Apple Peel Extracts (mDP). Phloroglucinol degradation (Phloroglucinolysis) was used for the estimation of *mDP* and procyanidin contents according to Karonen and co-workers (*25*). Briefly, 10 mg of apple extract was dissolved in 2 mL of a solution of 0.1 M HCl in methanol containing 50 mg/mL phloroglucinol and 10 mg/mL ascorbic acid. The reaction mixture was incubated at 50 °C for 20 min, and then 10 mL of 40 mM aqueous sodium acetate was added to stop the reaction. For fresh apple peel analysis, 100 mg was extracted in 1.0 mL of 70% actetone/water (v/v) containing 10 mg/mL of ascorbic acid. Samples were vortexed to mix thoroughly and sonicated by 20 min, before being centrifuged for 10 min (14000*g*). A 100 μL aliquot was evaporated to dryness under vacuum prior to phloroglucinolysis. Additionally, *mDP* was obtained by means of thiolysis with toluene-α-thiol and cysteamine (*26, 27*). Phloroglucinolysis products were analyzed by the same RP-HPLC described above. Quantitative determination of (−)-epicatechin, (+)-catechin, and the degradation products was performed using external standards. The calibration plots for (−)-epicatechin and (+)-catechin showed a linear range from 2−20 μg/mL ($r^2 = 0.9999$) and 10−60 μg/mL ($r^2 = 0.9997$), respectively. The procyanidin content was calculated by summing the mass of all subunits (excluding the phloroglucinol portion of the phloroglucinol adducts). To calculate the mean degree of polymerization, the sum of extension subunits was divided by the sum of terminal subunits. The undegraded medium was used to quantify native (−)-epicatechin and (+)-catechin in the extracts and fractions.

Normal-Phase High Performance Chromatography. APPE, HMW, and LMW procyanidins were separated by normal-phase HPLC (NP-HPLC) according to Gu and co-workers (*28*) with a 250 × 4.6 mm (5 μm) Lichrospher 100 Diol column (Merck, Darmstadt, Germany). The solvent system was composed by solvent A (acetonitrile/acetic acid, 98:2 v/v) and solvent B (methanol/water/acetic acid, 95:3:2 v/v). Procyanidins were eluted with the following gradient system: 0−35 min, 0−40% B; 35−55 min; isocratic 40% B; 55−60; 40−0% B with 5 min of column reconditioning at a flow rate of 0.6 mL/min. Elution was monitored by fluorescence detection with excitation at 230 nm and emission detection at 321 nm. Peak assignment was done in comparison with the literature (*28*). Oligomers up to undecamers were estimated by NP-HPLC.

Microplate Urease Test. Evaluation of urease activity was done according to a previously published methodology (*29*). *H. pylori* and *Canavalia ensiformis* ureases (Jack bean urease; E.C.3.5.1.5; Sigma-Aldrich), were used in the assay mixture (25 μL, 4 U) with 25 μL of different concentrations of polyphenols. Samples were preincubated for 0.5−4 h at room temperature in a 96-well assay plate. After preincubation, 200 μL of 100 mM phosphate buffer at pH 6.8 containing 150 mM urea and 0,002% phenol red were added, and changes in absorbance at 570 nm were measured by a micro plate reader Synergy HT (BioTek).

In-Gel Urease Activity. For in-gel detection of urease activity, two procedures were used. First, 25 μL of *H. pylori* urease (10 μg total proteins) was used in the assay mixture with 25 μL of different concentrations of polyphenols. Samples were preincubated for 1 h at room temperature. After preincubation, 10 μL were loaded and separated by electrophoresis under nondenaturing conditions with a 7% polyacrylamide gel in a Mini-protean III system apparatus (BioRad, USA). Electrophoresis was performed at 100 V in a Tris-glycine buffer system containing Tris HCl (25 mM) and glycine (250 mM) at pH 8.8. After electrophoresis, gels were washed sequentially with cold acetate buffer (5 μM, three times) and once with bidistilled water. In-gel activity was immediately determined according to the procedure previously described by Mobley and co-workers (*30*). Briefly, after the washing step, the gel was placed in a 0.02% cresol red−0.1% EDTA (pH 6.7). The procedure was repeated until the gel remained yellow and incubated with 150 mM urea at 37 °C until pink-reddish bands appeared.

With the aim to avoid incubations with the substrate and cresol red in a liquid medium as in the original protocol, some modifications were included. For this purpose, a thin film of agarose was prepared daily using the casting frame of the Miniprotean III system, with the same glass cassette used for PAGE (0.75 mm). Agarose (80 mg) was suspended in 0.02% cresol red−0.1% EDTA (pH 6.7), containing 150 mM urea. Agarose was melted using a conventional microwave oven and immediately loaded into the gel cassette. For this purpose, the coomb was omitted, and the agarose thin film was cooled at room temperature and stored at 4 °C until use. Polyphenols from apple peel were assayed in the concentration range of 0−1000 μg/mL. Under gentle agitation, the gels were incubated with the polyphenols (25 mL, 1 h at 25 °C). After the incubation period, samples (extracts or buffer) were carefully drained, and the gels were carefully deposited over a transparent plastic sheet. The agarose film was put in contact with the polyacrylamide gel. Another plastic sheet was used to cover the agarose film, forming a sandwich. The entire process was carried out onto the surface of a scanner (Snapscan e20, Agfa) allowing the record of band appearance.

RESULTS AND DISCUSSION

Apple Peel Extracts Characterization. Quantitative analysis based on the RP-HPLC polyphenolic profile indicates that quercetin glycosides account for about 58% of the total polyphenols present in APPE (**Table 1**; **Figure 1A**). As shown in the APPE chromatographic profile, the major quercetin glycosides identified were rutin, hyperoside, isoquercitrin, two quercetin-3-*O*-pentosides, and quercitrin. This chromatographic profile is remarkably similar to that obtained in the fresh peel of Granny Smith apples (upper chromatographic profile in **Figure 1A**, **Table 1**). The latter result suggests that the procedures of extraction and subsequent absorption of apple peel polyphenols did not modify the fresh apple peel profile generating artifacts, loss of compounds, or selective enrichment. Interestingly, the flavonoids found in this study for APPE and fresh Granny Smith apple peel are essentially the same as those reported previously to occur in apple peel from Granny Smith and from other apple varieties (*31*).

Because of its better sensitivity and selectivity, flavan-3-ols and procyanidins B1, B2, and C1 present in APPE were analyzed by HPLC-FLD. As depicted in **Figure 1B**, flavan-3-ols are represented mainly by epicatechin and only traces of catechin. Total procyanidins in APPE were estimated by phloroglucinolysis, accounting for 24.38% of the total polyphenol content (**Table 1**). Procyanidin B1, B2, and C1 content in

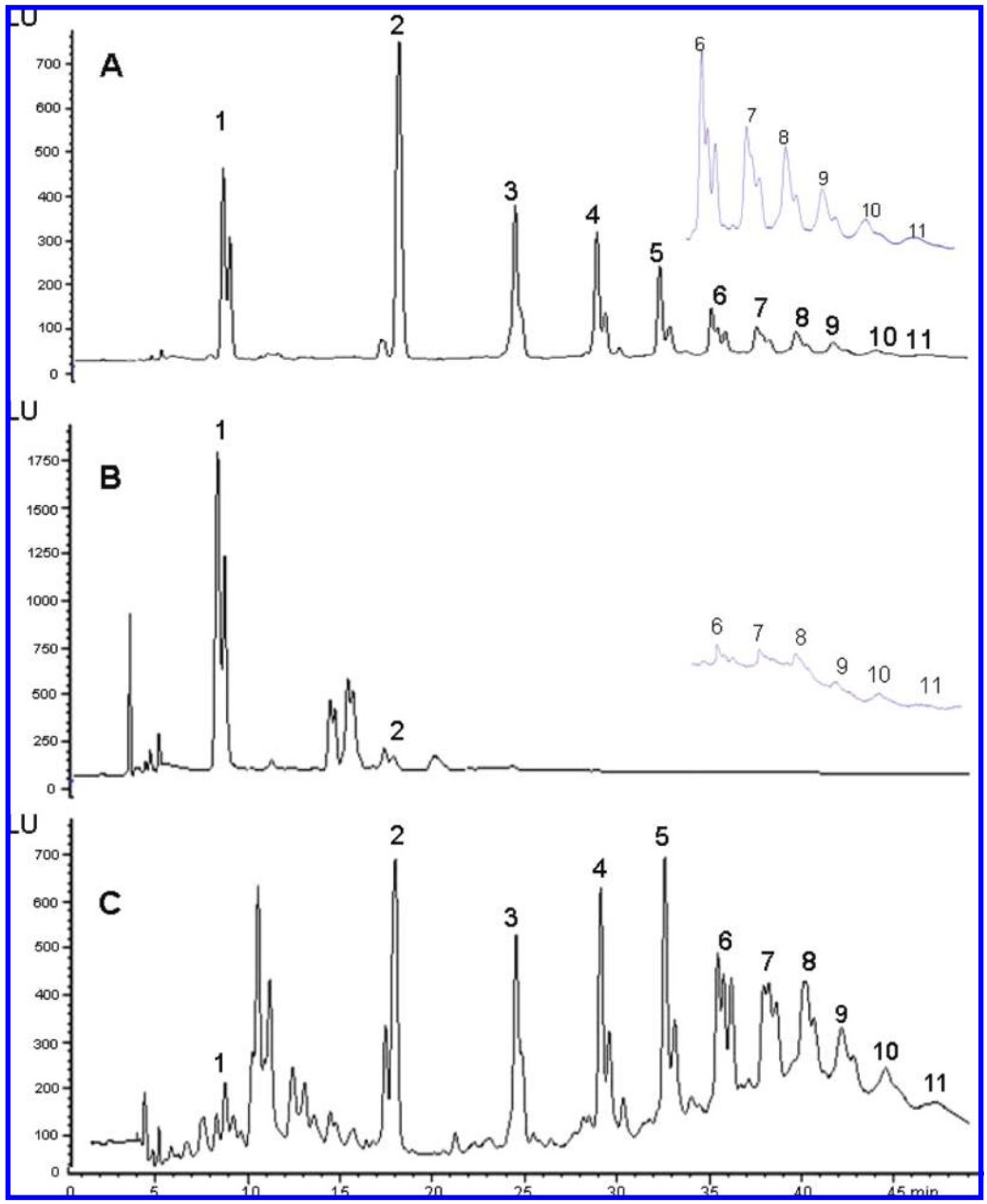

Figure 3. Normal-phase HPLC fluorescence trace of procyanidins from apple peel extracts. (**A**) APPE, (**B**) LMW, and (**C**) HMW. The numbers beside the peaks indicate the degree of polymerization of B-type procyanidins.

APPE evaluated by RP-HPLC represented 5.38% of the total polyphenols. These values suggest that the main procyanidins in APPE belong to the high MW group. Indeed the analysis of APPE by NP-HPLC showed that oligomeric material up to undecamers represented around 19.0% of the APPE total polyphenol content (**Figure 3A**). The contribution of phenol carboxylic acids (calculated as caffeic acid) and chalcones (calculate as phloridzin) reached 0.9 and 12%, respectively.

With the aim to fractionate APPE polyphenols according to their molecular size, a combination of Sephadex LH-20 and Toyopearl HW-40s column chromatography was used. As result of this strategy, low molecular weight (LMW) and high molecular weight (HMW) polyphenolic fractions were obtained, which was preliminarily confirmed by TLC analysis (**Figure 1C**). Polymeric and oligomeric procyanidins were exclusively concentrated in the fraction named HMW and under TLC examination appear as a poorly separated group of compounds. Polyphenols from LMW were visualized as better separated spots with Rfs $\geq$0.5. It should be noted that DMACA staining allows a selective detection of flavan-3-ol structures (monomers and polymers), and after 15−20 min, they appear as blue to green spots. As depicted in **Figure 1C**, flavan-3-ol (monomers and some dimers) are also present in the LMW fraction along with the quercetin glycosides (orange to brown spots) previously identified by HPLC-DAD. Therefore, by means of the fractionation procedure, polyphenols from APPE were pooled only according to their molecular size.

Determination of the *mDP* of APPE, HMW, and LMW was carried out by depolymerization via phloroglucinolysis. The values were calculated from the HPLC-FLD chromatographic profiles (**Table 2**). Although the three samples possess a distinctive degree of polymerization, total polyphenolic contents were very similar (500−600 mg GAE/g). **Figure 2** presents the RP-HPLC chromatographic profile of procyanidins subjected to depolymerization with phloroglucinol. By means of this procedure, it is possible to obtain results similar to those of the depolymerization with toluene-α-thiol and cysteamine (data not shown), in a shorter time and without the toxicity problems

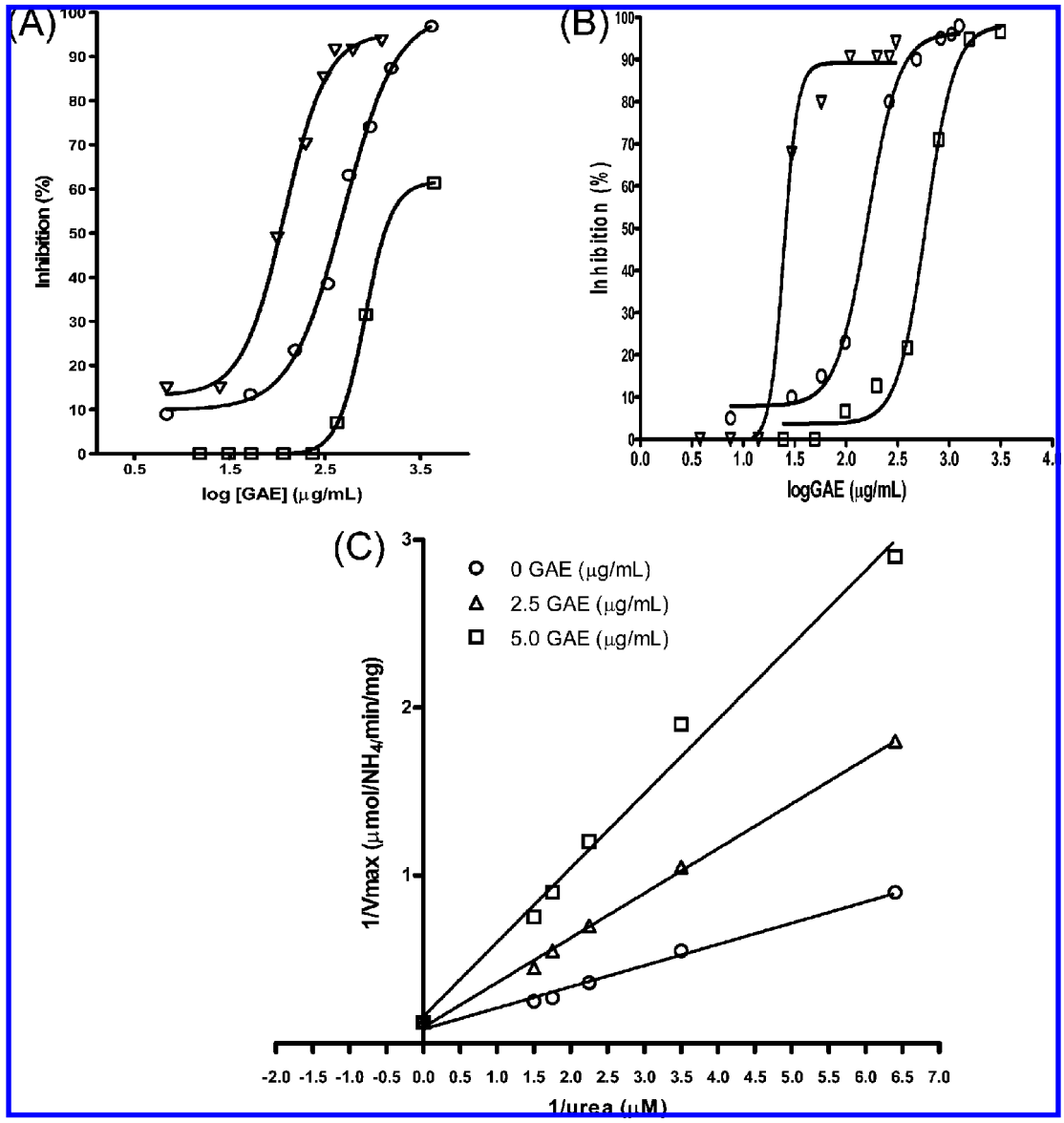

Figure 4. Inhibition of urease by APPE, HMW, and LMW. (**A**) Semilog graphic of *H. pylori* urease inhibition by apple peel polyphenols. (**B**) Semilog graphic of Jack bean urease inhibition by apple peel polyphenols. APPE, -○-; HMW, -▽-; LMW, -□-. (**C**) Lineweaver-Burke plots showing 1/urease activity and 1/substrate concentration. Data in the presence of 0 (○); 2.5 (△); and 5 (□) GAE (μg/mL) of APPE are presented as the mean of four determinations.

associated with the previous one. The epicatechin interconversion to catechin is not an important reaction in this procedure. Indeed, catechin levels detected in the samples subjected to degradation with phloroglucinol were the same as those in the samples without treatment (not shown). Depolymerization with phloroglucinol generated profiles where it is clearly seen that the main extension and terminal unit was epicatechin (**Figure 2A**,**B**). The latter eluted with a t_R = 11.5 min, whereas the nucleophile−epicatechin adduct did at t_R = 6.8 min. After a detailed analysis of the HPLC profile, it was not possible to find the nucleophile−catechin peak. The latter usually appears as a partially resolved peak before the epicatechin−adduct peak. Considering all of these results, it is suggested that in the three extracts, procyanidins would be mainly derived from epicatechin and, in a smaller degree, from catechin just as it has been previously informed for fresh apple peel (*32*). It is necessary to notice that the depolymerization profiles corresponding to the analysis of fresh apple peel were identical to those previously described for APPE (**Table 1**).

Figure 3 shows the result of the NP-HPLC separation of APPE procyanidin oligomers and the fractions LMW and HMW. Profiles depicted in **Figure 3A−C** show procyanidin peaks or clusters with different molecular weight. This complexity would correspond to the possible rotamer combinations, as reported by other authors (*33*). By means of the study of NP-HPLC profiles, it is possible to confirm that LMW possesses mostly monomeric units (*mDP* = 1). APPE possesses an *mDP* = 2−4, while for HMW *mDP* = 8−10.

Effects of APPE, LMW, and HMW on Urease Activity. The effect of APPE and its LMW and HMW fractions on urease activity was studied using *H. pylori* and Jack bean (for comparative purposes) as sources for the enzyme. Jack bean urease has been widely used as an enzyme model for inhibitor screening (*29*). Importantly, in the case of *H. pylori*, its α subunit shares 48% of amino acid sequence identity with the corresponding N-terminal sequences of Jack bean urease (*34*). As depicted in **Figure 4A−C**, the whole extract and its low and high MW fractions were all active in promoting an inhibition

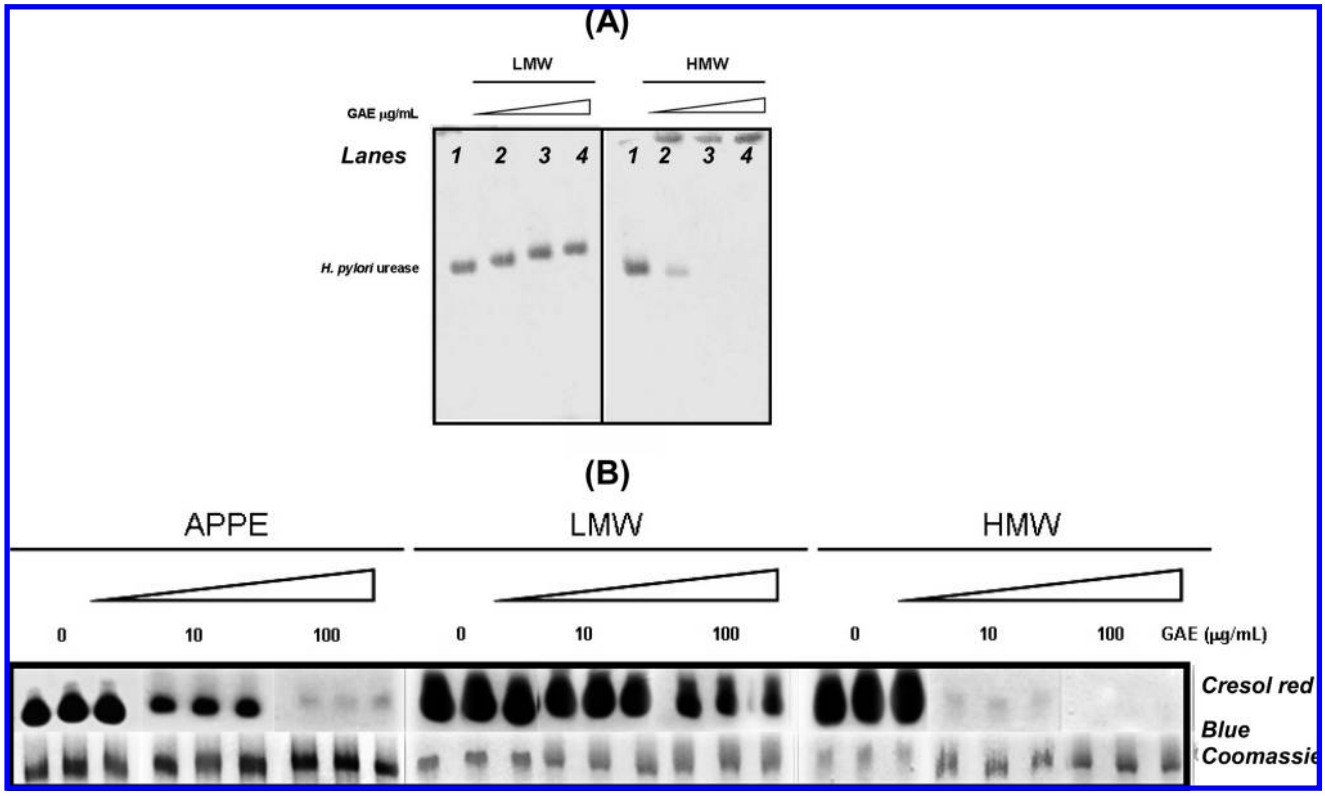

Figure 5. Effects of apple peel fractions upon urease activity detected in-gel. Nondenaturant gel electrophoresis of urease extracted from *H. pylori* on 7% polyacrilamide. (**A**) Before electrophoresis, apple peel fractions LMW and HMW were previously incubated with *H. pylori* urease as described in Material and Methods. Lane 1 = control urease; lanes 2, 3, and 4 = LMW = HMW = 100−400−800 GAE μg/mL. (**B**) Effects of apple peel crude and fractionated extracts upon *H. pylori urease* activity by a modified in-gel procedure. APPE, LMW, and HMW effect upon *H. pylori* urease activity in nondenaturant gels as detected by agarose−urea−cresol red film procedure. The same gels stained by Coomassie blue are presented on the bottom panel. Figures are representative of three independent experiments.

of urease activity. Since the main feature of apple peel polyphenols is that of acting as antioxidants, the comparison of their effect on urease activity was done on the basis of using micrograms of gallic acid as mass equivalent (GAE) for the distinct tested preparation. The inhibitory effects were in all cases concentration-dependent. **Table 2** shows the IC_{50} for APPE and its fractions upon *H. pylori* and Jack bean ureases. For *H. pylori* urease, the IC_{50} values were 119, 800, and 516 μg GAE/mL, for HMW, LMW and APPE, respectively. The low solubility of LMW fraction precluded assaying higher concentrations. For Jack bean urease, the IC_{50} values were 103, 594, and 180 μg GAE/mL for HMW, LMW, and APPE, respectively. On the basis of the IC_{50} ranking order and level of GAE, these results suggest that the urease-inhibitory activity displayed by APPE is associated, primarily, with the presence of high MW polyphenols and, to a lower degree, with the presence of monomeric components. Furthermore, the close similarity in both the shape and slope of the semilog curves describing the effect of HMW and that of APPE suggests that the inhibition of urease induced by the latter would indeed be attributable to the presence of high MW components (**Figure 4A−C**).

The slightly smaller IC_{50} values obtained with the Jack bean (compared with *H. pylori*) urease could relate to a higher purity, which would result in a lower nonspecific binding of polyphenols to the former enzyme. Urease, extracted from *H. pylori* cultures, has been reported to copurify with other proteins such as GroEL, IlvC, HsPB, and Hsp60 (*35*). Indeed, SDS−PAGE run by us for the *H. pylori* lysates revealed the presence of certain bands that lacked ureolytic activity (data not shown).

Effects of APPE, LMW, and HMW on In-Gel Urease Activity. A decrease in the urease activity was observed after incubation with apple peel extracts. To better understand this activity, the effect of apple peel fractions over urease electrophoretic mobility was evaluated. As shown in **Figure 5A**, HMW concentration-dependently generated insoluble aggregates, which resulted from the formation of procyanidin−urease complexes. LMW also showed this kind of interaction but to a lesser degree. These high molecular weight complexes (**Figure 5A**, lanes 2−4), were unable to migrate through the 7% polyacrylamide gel under nondenaturing conditions. Although the aggregate formation occurred, urease activity was maintained as it is possible to evidence it after the in-gel detection with cresol red. Apparently, this observation is not concordant with the concentration-dependent inhibition observed in the 96-well format (**Figure 4**). This difference can be due to the methodological difference between both assays. For instance, in the in-well assay the urease is continually exposed to the tested polyphenols, meanwhile in the in-gel evaluation, the polyphenols could be removed from the aggregate during the conventional steps after the electrophoretic run, i.e., washing the gel in a continuous form with acetate buffer (pH 6.8), as a necessary condition to allow the subsequent addition of cresol red. Because of this difference, both methodologies are not comparable. Additionally, the results in-well (enzyme kinetics) suggested a competitive and reversible inhibition (**Figures 4B**); therefore, it is possible to conclude that the in-gel activity detection would not be the best procedure to evaluate this kind of urease inhibitors. Hence, a modification to the original protocol was carried out, in which the enzyme preparation (*H. pylori* urease) was first subjected

to a PAGE separation (under nondenaturing conditions), then gel strips (with urease activity previously established) were initially incubated with increasing concentrations (expressed as GAE) of APPE, HMW, or LMW, and removed from such solutions afterward. Thereafter, strips were subsequently covered with an agarose film containing urea (150 mM) and cresol red, which allowed revealing in a semiquantitative form, the remaining urease activity (**Figure 5B**). With this procedure, the washing steps previously referred to as the possible cause of urease reactivation were completely avoided. **Figure 5B** showed a clear inhibitory effect of HMW against urease from a concentration of 10 μg GAE/mL. Contrarily, APPE showed only a slight enzyme inhibition at the same concentration, this is consistent with the proposal that molecules (most likely procyanidin) with a higher degree of polymerization underlie the urease-inhibitory properties of apple peel extracts. However, when the concentration was increased up to 100 μg GAE/mL, both preparations achieved almost a complete inhibition. The inhibitory effect of such preparations is not attributable to differences in the protein concentration loaded in the gels (**Figure 5B**, inferior panel). These results using this methodological approach were consistent with the concentration-dependence results obtained previously in the in-well assay. Moreover, a significant restoration of urease activity was seen when agarose films containing a higher concentration or urea (300 mM) were used. Interestingly, in the latter assay, the concentration needed to achieve total urease inhibition was substantially higher than the one estimated with the improved in-gel assay. This may suggest that electrophoretic separation of the native urease would result in a lower nonspecific binding of polyphenols to other *H. pylori* proteins.

In comparison to HMW, apple peel polyphenols, containing low MW polyphenols, showed a 6- to 7-fold lower effectiveness to inhibit *H. pylori* urease. Hitherto, only a few studies have reported an antiurease effect of low MW polyphenols. Xiao and co-workers (*22*) evaluated the *H. pylori* antiurease activity of 20 synthetic polyphenols based on isoflavones. The presence of two ortho hydroxyl groups and the integrity of the C-ring were essential for their inhibitory activity. The urease inhibition induced by these synthetic polyphenols was time-dependent and in some cases was totally reverted by the subsequent addition of the thiol agents, DTT, or β-mercaptoethanol. Contrarily in this research, the urease-inhibitory effect displayed by APPE, HMW, and LMW was not time-dependent and only partially (less than 10%) reverted by DTT or β-mercaptoethanol (data not shown).

Regarding polymerization degree, hops and red wine extracts with demonstrated in vivo anti-*H. pylori* effects (*36*, *37*) have established a structure−activity relationship between the degree of polymerization of the polyphenols present in these extracts and the degree of inactivation of VacA, another important virulence factor. Only a few studies have evaluated the effect of some dimeric procyanidins (B1 and B2) on urease activity (*38*). Unfortunately, these studies did not address the importance of the polymerization degree with regard to the inhibitory effect of such procyanidins. In APPE, quercetin glycosides (~58%) are more abundant than procyanidins (~25%), which is particularly interesting because of the results reported herein. Although the HMW compounds account for a great part of *H. pylori* urease inhibition, further investigation is needed to establish if LMW (quercetin glycosides) could interact with other molecular targets decreasing *H. pylori* viability. Urease neutralization not only precludes the *H. pylori* colonization ability but also the synthesis of pro-inflammatory cytokines such as interleukin-8 (IL-8) (*39*). In gastric epithelial cells, IL-8 production is mediated by NF-κB. The latter activation could be induced by *H. pylori* urease binding to CD74 (invariant chain of the mayor histocompatibility complex II, MHC II). Therefore, APPE constituents could also afford benefits by ameliorating the inflammatory damage caused by *H. pylori* infection. In our laboratory, APPE is currently investigated considering other molecular targets relevant to the *H. pylori* viability and certain pathways associated with host gastric mucosa damage.

In summary, in this work we found that APPE inhibited the *H. pylori* urease in vitro. APPE effectively blocked the activity of isolated urease, an interesting effect with respect to *H. pylori* colonization capability. In this research, we successfully identified high molecular weight polyphenols as the main constituents associated with urease inhibition.

ACKNOWLEDGMENT

E.P. thanks Isabel Mella (Laboratory of Microbiology, Institute of Nutrition and Food Technology, University of Chile) for technical assistance.

LITERATURE CITED

(1) Boyer, J.; Hai Lui, R. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, *3*, 5–20.
(2) Wolfe, K.; Liu, R. H. Apple peels as a value-added food ingredient. *J. Agric. Food Chem.* **2003**, *51*, 1676–1683.
(3) Lachman, J.; Šulc, M.; Sus, J.; Pavlíková, O. Polyphenol content and antiradical activity in different apple varieties. *Scientia Horticulturae.* **2006**, *6*, 95–102.
(4) Barth, S. W.; Fähndrich, C.; Bub, A.; Dietrich, H.; Watzl, B.; Will, F.; Briviba, K.; Rechkemmer, G. Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis* **2005**, *8*, 1414–1421.
(5) Veeriah, S.; Hofmann, T.; Geli, M.; Dietrich, H.; Will, F.; Schreier, P.; Knaup, B.; Pool-Zobel, B. L. Apple polyphenols and products formed in the gut differently inhibit survival of human cell lines derived from colon adenoma (LT97) and carcinoma (HT29). *J. Agric. Food Chem.* **2007**, *55*, 2892–2900.
(6) Kern, M.; Pahlke, G.; Balavenkatraman, K. K.; Böhmer, F. D.; Marko, D. Apple polyphenols affect protein kinase C activity and the onset of apoptosis in human colon carcinoma cells. *J. Agric. Food Chem.* **2007**, *55*, 4999–5006.
(7) Fridrich, D.; Kern, M.; Gudrun, P.; Volz, N.; Will, F.; Dietrich, H.; Marko, D. Apple polyphenols dimish the phosphorylation of the epidermal growth factor receptor in HT29 colon carcinoma cells. *Mol. Nutr. Food Res.* **2007**, *51*, 594–601.
(8) Fini, L.; Selgrad, M.; Fogliano, V.; Graziani, G.; Romano, M.; Hotchkiss, E.; Daoud, Y. A.; De Vol, E. B.; Boland, R.; Riccardiello, L. Annurca apple polyphenols have potent demethylating activity and can reactivate silenced tumor supresor genes in colorectal cancer cells. *J. Nutr.* **2007**, *137*, 2622–2628.
(9) McGhie, T.; Hunt, M.; Barnett, L. Cultivar and growing region determine the antioxidant polyphenolic concentration and composition of apples grown in New Zealand. *J. Agric. Food Chem.* **2005**, *53*, 3065–3070.
(10) Hamauzu, Y.; Inno, T.; Kume, Ch.; Irie, M.; Hiramatsu, K. Antioxidant and antiulcerative properties of phenolics from Chinese quince, quince, and apple fruits. *J. Agric. Food Chem.* **2006**, *54*, 765–772.
(11) Tsao, R.; Yang, R.; Xie, R.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple. *J. Agric. Food Chem.* **2005**, *53*, 4989–4995.
(12) Graziani, G.; D'Argento, G. D.; Tuccillo, C.; Loguercio, C.; Ritieni, A.; Morisco, F.; Del Vecchio, C.; Fogliano, V.; Romano, M. Apple polyphenols extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. *Gut* **2005**, *2*, 193–200.

(13) D'Argenio, G.; Mazzone, G.; Tuccillo, C.; Grandote, I.; Gravina, A. G.; Graziani, G.; Fogliano, V.; Romano, M. Apple polyphenol extracts prevent aspirin-induced damage to the rat gastric mucosa. *Br. J. Nutr.* **2008**, *16*, 1–9.
(14) Hamauzu, Y.; Irie, M.; Kondo, M.; Fujita, T. Antiulcerative properties of crude polyphenols and juice of apple, and Chinese quince extracts. *Food Chem.* **2008**, *108*, 488–495.
(15) Hamauzu, Y.; Forest, F.; Hiramatsu, K.; Sugimoto, M. Effect of pear (*Pyrus communis* L.) procyanidins on gastric lesions induced by HCl/ethanol in rats. *Food Chem.* **2007**, *100*, 255–263.
(16) Golding, J. B.; McGlasson, W. B.; Wyllie, S. G.; Leach, D. N. Fate of apple peel phenolics during cool storage. *J. Agric. Food Chem.* **2001**, *49*, 2283–2289.
(17) Dunn, B. E.; Cohen, H.; Blaser, M. J. *Helicobacter pylori*. *Clin. Microbiol. Rev.* **1997**, *10*, 720–741.
(18) Atherton, J. C. The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu. Rev. Pathol.: Mech. Dis.* **2006**, *1*, 63–96.
(19) Franco, A. T.; Johnston, E.; Krishna, U.; Yamaoka, Y.; Israel, D. A.; Nagy, T. A.; Wroblewski, L. E.; Piazuelo, M. B.; Correa, P. Regulation of gastric carcinogenesis by *Helicobacter pylori* virulence factors. *Cancer Res.* **2008**, *2*, 379–387.
(20) Mobley, H. L. T.; Hu, L. T.; Foxall, P. A. *Helicobacter pylori* urease: properties and role in pathogenesis. *Scand. J. Gastroenterol.* **2001**, *26*, 39–46.
(21) Sun, B.; Belchior, P.; da-Silva, R.; Spranger, M. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr., A.* **1999**, *841*, 115–121.
(22) Xiao, Z.-P.; Shi, D.-H.; Li, H.-Q.; Zhang, L.-N.; Xu, Ch.; Zhu, H.-L. Polyphenols based on isoflavones as inhibitors of *Helicobacter pylori* urease. *Bioorg. Med. Chem.* **2007**, *15*, 3703–3710.
(23) Weatherburn, M. W. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **1967**, *39*, 971–974.
(24) Jeréz, M.; Touriño, S.; Sineiro, J.; Torres, J. L.; Núñez, M. J. Procyanidins from pine bark: Relationship between structure, composition and antirradical activity. *Food Chem.* **2007**, *104*, 518–527.
(25) Karonen, M.; Leikas, A.; Loponen, J.; Sinkkonen, J.; Ossipov, V.; Pihlaja, K. Reversed-phase HPLC-ESI/MS analysis of Birch leaf proanthocyanidins after their acidic degradation in the presence of nucleophiles. *Phytochem. Anal.* **2007**, *18*, 378–386.
(26) Monagas, M.; Garrido, I.; Bartolomé, B.; Gómez-Cordovés, C. Chemical characterization of commercial dietary ingredients from *Vitis vinifera* L. *Anal. Chem. Acta.* **2006**, *563*, 401–410.
(27) Selga, A.; Torres, J. L. Efficient preparation of catechin thio conjugates by one step extraction/depolymerization of pine (*Pinus pinaster*) bark procyanidins. *J. Agric. Food Chem.* **2005**, *53*, 7760–7765.
(28) Gu, L.; Hager, A. C.; Robbins, R. J.; Prior, R. L. Analyses of procyanidins in foods using diol phase HPLC. *FASEB J.* **2008**, *22*, 148.5.
(29) Tanaka, T.; Kawase, M.; Tani, S. α-hydroxyketones as inhibitors of urease. *Bioorg. Med. Chem.* **2004**, *12*, 501–505.
(30) Mobley, H. L. T.; Cortesia, M. J.; Rosenthal, L. E.; Jones, B. D. Characterization of urease from *Campylobacter pylori*. *J. Clin. Microbiol.* **1988**, *26*, 831–836.
(31) Zessner, H.; Pan, L.; Will, F.; Klimo, K.; Kanuft, J.; Niewöhner, R.; Hümmer, W.; Owen, R.; Richling, E.; Frank, N.; Schreier, P.; Becker, H.; Gerhauser, C. Fractionation of polyphenols-enriched apple peel juice extracts to identify constituents with cancer chemopreventive potential. *Mol. Nutr. Food Res.* **2008**, *52*, 28–44.
(32) Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *1*, 14–20.
(33) Tarascou, I.; Duchase, M. A.; Dufourc, E. J.; Moskau, D.; Fouket, E.; Laguerre, M.; Pianet, I. Structural and conformational analysis of two native procyanidins trimers. *Magn. Reson. Chem.* **2007**, *45*, 157–166.
(34) Krajewska, B.; Zaborska, W. Jack bean urease: The effect of active-site binding inhibitors on the reactivity of enzyme thiol groups. *Bioorg. Chem.* **2007**, *5*, 355–365.
(35) Pyndiah, S.; Lasserre, J. P.; Menard, A.; Claverol, S.; Prouzet-Mauleon, V.; Megraud, F.; Zerbib, F.; Bonneu, M. Two-dimensional blue native/SDS gel electrophoresis of multiprotein complexes from *Helicobacter pylori*. *Mol. Cell Proteomics* **2007**, *6*, 193–206.
(36) Yahiro, K.; Shirasaka, D.; Tagashira, M.; Wada, A.; Morinaga, N.; Kuroda, F.; Choi, O. Inhibitory effects of polyphenols on gastric injury by *Helicobacter pylori* VacA toxin. *Helicobacter* **2005**, *3*, 231–239.
(37) Ruggiero, P.; Rossi, G.; Tombola, F.; Pancotto, L.; Lauretti, L.; Del Giudice, G.; Zoratti, M. Red wine and green tea reduces *H. pylori*-or VacA-induced gastritis in a mouse model. *World J. Gastroenterol.* **2007**, *3*, 349–354.
(38) Lee, J. S.; Um, H. Ch.; Hahm, K.; Park, Ch. S.; Kang, D.; Surh, Y. Natural urease inhibitors. *Cancer Prev. Res.* **2006**, *2*, 129–136.
(39) Beswik, E. J.; Pinchuk, I. V.; Minch, K.; Suárez, G.; Sierra, J. C.; Yamaoka, Y.; Reyes, V. E. The *Helicobacter pylori* urease B subunit binds to CD74 on gastric epithelial cells and induces NF-κB activation and interleukin-8 production. *Infect. Immun.* **2006**, *2*, 1148–1155.

Received for review August 20, 2008. Revised manuscript received November 12, 2008. Accepted November 18, 2008. This study was partially supported by Comisión Nacional de Ciencia y Tecnología of Chile (Grant No. 24080071).

JF8025698