Analysis of Phenolic Acids in Barley by High-Performance Liquid Chromatography

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Phenolic acids from 30 barley varieties (combination of hulled/hulless/two-row/six-row/regular/waxy) were investigated by HPLC following four different sample treatments: (a) simple hot water extraction, (b) extraction after acid hydrolysis, (c) acid plus α-amylase hydrolysis, and (d) acid plus α-amylase plus cellulase hydrolysis treatments. The benzoic acid (*p*-hydroxybenzoic, vanillic, and protocatechuic acids) and cinnamic acid derivatives (coumaric, caffeic, ferulic, and chlorogenic acids) were identified, and some of the phenolic acids were quantified after each above-mentioned treatment. The data indicated that a combination of sequential acid, α-amylase, and cellulase hydrolysis treatments might be applicable for release of more phenolic acids from barley.

Keywords: *Phenolic acids; benzoic acid; caffeic acid; barley; HPLC*

INTRODUCTION

The identification and quantitative analysis of phenolic acids in plants are necessary because of their importance as substrates in the biosynthesis of aromatic amino acids in plants (*1*). Phenolic acids also influence the flavor, taste, and color of foods. In recent years, many phenolic compounds have attracted the attention of food and medical scientists because of their antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and their capacity to modulate some key cellular enzyme functions (*2*).

Cereals contain a wide range of phenolic acids, particularly those belonging to the benzoic and cinnamic acids series. Phenolic acids occur widely in plants and are second only to the flavonoids in importance as secondary plant metabolites. They differ from other phenols by their acidic character. In the plant, phenolic acids can be esterified with other small molecules of aliphatic alcohols, phenols, phenolic acids, alcohols, and alkaloids. Such a group of compounds is the water-soluble pentosans, which are nonstarch polysaccharides of xylose, with randomly attached side groups of single or double arabinose units that in turn are esterified with phenolic acids. These ester compounds can be hydrolyzed by acid, and phenolic acids can be released.

Phenolic acids with their carboxyl and hydroxyl groups are capable of binding with starch and other polysaccharides through hydrogen bonds, chelation, or covalent bonds, forming bridges or cross-links (*2*). Therefore, using a combination of α-amylase and acid hydrolysis of starch can release phenolic acids that are bound with starch.

The outer layers of cereal grains (husk, pericarp, testa, and aleurone cells) contain the greatest concentrations of total phenolics, whereas their concentration is considerably lower in the endosperm layers (*3*). For instance, free and esterified phenolic acids were found in wheat, corn, rice, and oat (*4*). Some researchers considered phenolic acids to be concentrated in the cell walls of the outer layers, where they are mainly esterified to the arabinose side groups of arabinoxylans (*5*). However, others indicated that phenolic acids were mainly present in the aleurone layer and endosperm (*6*). Shibuya (*7*) reported the occurrence of high levels of diferulic acid in rice endosperm cell walls proportional to the level of arabinoxylans.

Ferulic acid is the major low molecular weight phenolic acid in many common cereals. It exists in the seed coat (*8*). Only trace amounts were found in the starchy endosperm (*9*). The presence of *trans*-ferulic, syringic, and vanillic acids was reported in wheat (*4*). Rice contained two additional phenolic compounds compared to wheat: *p*-hydroxybenzoic and protocatechuic acids. Oat and corn contained several different phenolic compounds: *p*-hydroxybenzoic, vanillic, protocatechuic, syringic, ferulic, caffeic, and sinapic acids (*4*). Hatcher and Kruger (*10*) also reported that wheat contained six phenolic acids, sinapic, ferulic, vanillic, syringic, caffeic, and coumaric acids, that were related to the ash content and color of milled flour.

Extraction of free phenolic acid and soluble phenolic acid esters for quantification by HPLC was carried out using organic solvents. Prior to extraction, entrapped phenolic acids were released by alkali or acid treatment (*4*, *11*, *12*). This procedure involved several steps and thus was quite time-consuming. Enzymatic treatments were also employed to release phenolic acid during sample preparation. Zupfer et al. (*13*) used α-amylase to degrade starch in order to release ferulic acid in barley. Their procedure included only two steps: acid and amylase hydrolysis that quantified the concentration of ferulic acid in the range of 343−580 μg/g. Bartolomé and Gómez-Cordovés (*14*) used commercial enzymes (Viscozyme L, Ultraflo L, Termamyl, and Lallzyme) to investigate the phenolic acids in barley spent grain. They found that Ultraflo L led to the highest release of both ferulic and *p*-coumaric acids from barley spent grain. Andreasen et al. (*15*) used com-

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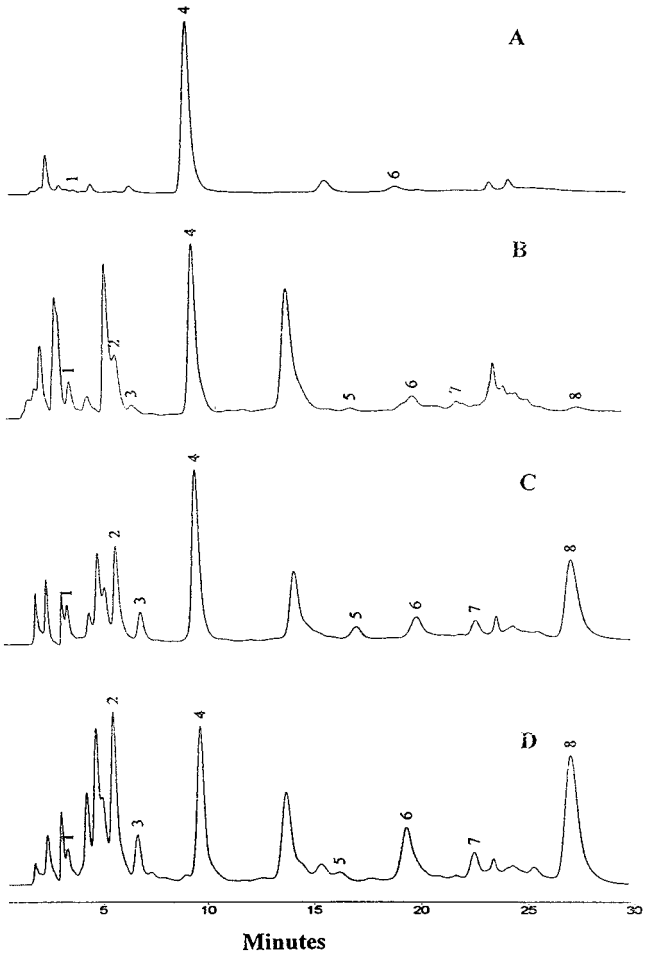

Figure 1. Chromatogram of phenolic acids in Candle barley after different treatments: (A) hot water extraction; (B) acid hydrolysis treatment; (C) acid and amylase hydrolysis; (D) acid, amylase, and cellulase hydrolysis. Peaks labeled 1−8 represent protocatechuic, *p*-hydroxybenzoic, vanillic, syringic (internal standard), caffeic, chlorogenic, *p*-coumaric, and ferulic acids, respectively.

mercial plant cell wall degrading enzyme (Macer, Grindamyl, Rapidase, and Viscozyme) preparations to release the phenolic acids in rye grain. Their results indicated that enzyme treatments significantly enhanced extraction of phenolic acids from rye meal. The above-mentioned studies showed that enzymatic release of phenolic acids could be employed for the efficient quantification of phenolic acids in cereals.

Although there are several studies (*3*, *6*, *13*) on barley phenolic acids, the identification and quantitation of all major phenolic acids in different barley varieties using pretreatments, such as acid, α-amylase, and cellulase hydrolysis, have not been done methodically. Therefore, the objectives of this study were to investigate and compare different treatments (a, hot water extraction for free phenolic acids; b, acid hydrolysis for simple esterified phenolic acids; and c, acid hydrolysis followed by α-amylase hydrolysis of starch and similar polysaccharides to release bound phenolic acids) for the quantitation of phenolic acids in different barley cultivars and to set up a more complete pretreatment procedure (d, acid and α-amylase hydrolysis followed by cellulase degradation of plant cell wall to release insoluble-bound phenolic acids) to determine the content of different phenolic acids in 30 barley varieties from a combination of hulled/hulless/two-row/six-row/regular/waxy cultivars.

Table 1. Free Phenolic Acids in Hot Water Extract of Different Barley Varieties[a]

	μg/g of dry matter	
variety	protocatechuic acid	chlorogenic acid
six-row regular-hulled		
B1602	trace	4.09 ± 0.05
CDC Sister	trace	5.93 ± 0.11
Tukwa	trace	5.23 ± 0.08
AC Lacombe	trace	4.76 ± 0.12
Kasota	trace	5.21 ± 0.10
Stander	trace	11.8 ± 0.03
Robust	trace	16.28 ± 0.13
Excel	trace	2.22 ± 0.11
two-row regular-hulled		
Harrington	trace	14.2 ± 0.31
Leduc	trace	4.29 ± 0.06
Stein	2.60 ± 0.22	11.46 ± 0.22
Manley	2.08 ± 0.13	8.6 ± 0.14
CDC Dolly	trace	8.07 ± 0.12
six-row regular-hulless		
HB 504	trace	7.38 ± 0.33
AC Bacon	trace	5.98 ± 0.26
Falcon	trace	8.61 ± 0.20
two-row regular-hulless		
CDC Richard	2.11 ± 0.22	4.28 ± 0.05
Condor	2.18 ± 0.04	5.78 ± 0.10
HB 313	trace	4.38 ± 0.09
HB 801	trace	6.96 ± 0.14
HB 803	trace	5.94 ± 0.26
SB 89497	trace	4.97 ± 0.07
CDC Dawn	2.08 ± 0.09	10.44 ± 0.19
HB 335	trace	13.13 ± 0.12
Phoenix	2.64 ± 0.06	3.51 ± 0.15
two-row waxy-hulless		
Candle	2.01 ± 0.13	3.28 ± 0.06
HB 805	2.23 ± 0.14	4.72 ± 0.21
HB 806	2.88 ± 0.25	4.41 ± 0.33
HB 807	2.34 ± 0.19	3.21 ± 0.28
HB 340	2.37 ± 0.10	10.94 ± 0.11

[a] Mean ± standard deviation based on triplicate determinations is reported.

MATERIALS AND METHODS

Materials. Barley grains were obtained from three locations in Canada (Alberta, Saskatoon, and Winnipeg). Thirty varieties/lines of barley were used: eight varieties of six-row regular-hulled (B1602, CDC Sister, Tukwa, AC Lacombe, Kasota, Stander, Robust, and Excel); five varieties of two-row regular-hulled (Harrington, Leduc, Stein, Manley, and CDC Dolly); three varieties of six-row regular-hulless (HB 504, AC Bacon, and Falcon); nine varieties of two-row regular-hulless (CDC Richard, Condor, HB 313, HB 801, HB 803, SB 89497, CDC Dawn, Phoenix, and HB 335); and five varieties of two-row waxy-hulless (Candle, HB 805, HB 806, HB 807, and HB 340). Grains were cleaned and milled in a cyclone sample mill equipped with a 0.5 mm screen (models 3010-030, Udy Corp., Ft. Collins, CO). All chemical reagents, enzymes (α-amylase from *Aspergillus oryzae* crude, 39 units/mg of solid, and cellulase from *Aspergillus niger*, 0.57 units/mg of solid), and phenolic acid standards were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. *Sample Preparation. (1) Hot Water Extraction.* Hot water extraction was performed according to the Kajimoto et al. (*16*) method. Ground samples (0.1 g) were mixed with 2 mL of hot water in test tubes and heated in a boiling water bath for 1 h. The samples were cooled at room temperature and centrifuged (model TC, Sorvall Co., DuPont, Newtown, CT) at 10000*g* for 10 min. The supernatant was analyzed by HPLC.

Table 2. Phenolic Acids in Acid Hydrolysate of Different Barley Varieties[a]

variety	μg/g of dry matter			
	protocatechuic acid	vanillic acid	chlorogenic acid	ferulic acid
six-row regular-hulled				
B1602	83.41 ± 2.10	42.36 ± 1.88	19.06 ± 1.03	35.97 ± 0.69
CDC Sister	81.79 ± 1.33	39.15 ± 1.09	30.16 ± 0.54	63.50 ± 0.52
Tukwa	73.25 ± 1.23	36.25 ± 1.16	32.79 ± 0.85	50.21 ± 0.77
AC Lacombe	80.41 ± 0.98	31.26 ± 1.57	19.17 ± 1.11	61.54 ± 0.23
Kasota	91.21 ± 1.56	33.47 ± 0.96	25.59 ± 1.41	68.38 ± 0.58
Stander	76.31 ± 3.12	32.19 ± 2.41	56.51 ± 0.58	38.73 ± 0.91
Robust	71.38 ± 2.26	33.77 ± 1.63	78.05 ± 1.31	46.06 ± 1.10
Excel	82.07 ± 1.47	29.81 ± 1.89	9.93 ± 1.59	46.58 ± 0.49
av[b]	79.98c	34.78c	33.91d	51.37b
two-row regular-hulled				
Harrington	80.58 ± 0.69	41.94 ± 0.21	82.06 ± 0.82	18.90 ± 1.59
Leduc	68.23 ± 1.89	36.23 ± 0.98	24.05 ± 0.59	28.97 ± 0.71
Stein	87.44 ± 3.6	35.16 ± 2.19	43.07 ± 0.25	26.70 ± 0.52
Manley	65.79 ± 2.58	49.56 ± 0.78	46.69 ± 0.86	43.94 ± 0.33
CDC Dolly	75.87 ± 2.41	41.23 ± 1.56	40.73 ± 1.09	45.90 ± 0.85
av	75.58d	40.82a	47.32c	32.88d
six-row regular-hulless				
HB 504	74.89 ± 1.68	37.19 ± 2.65	50.50 ± 0.55	62.06 ± 0.19
AC Bacon	78.71 ± 2.45	39.66 ± 1.22	32.06 ± 0.29	46.51 ± 0.81
Falcon	86.54 ± 1.29	35.14 ± 0.89	73.11 ± 0.91	30.37 ± 0.47
av	80.05c	37.33b	51.89b	46.31c
two-row regular-hulless				
CDC Richard	89.55 ± 3.21	21.33 ± 1.21	31.68 ± 0.85	12.51 ± 0.59
Condor	88.01 ± 1.33	33.18 ± 0.48	35.54 ± 2.19	13.55 ± 0.90
HB 313	86.99 ± 1.46	29.66 ± 1.32	55.97 ± 1.25	21.17 ± 0.25
HB 801	86.44 ± 2.09	19.63 ± 1.54	49.15 ± 1.09	22.72 ± 0.58
HB 803	90.90 ± 1.26	8.66 ± 2.06	32.38 ± 1.81	36.78 ± 0.75
SB 89497	101.32 ± 2.53	15.31 ± 1.41	33.46 ± 0.59	36.54 ± 0.81
CDC Dawn	66.75 ± 0.59	12.33 ± 0.84	49.54 ± 0.87	45.35 ± 0.37
HB 335	88.86 ± 2.67	10.96 ± 0.77	127.24 ± 0.67	68.71 ± 1.20
Phoenix	85.27 ± 1.98	17.8 ± 1.81	55.52 ± 0.96	10.28 ± 1.09
av	87.12b	18.76e	52.28b	29.73e
two-row waxy-hulless				
Candle	80.26 ± 1.66	27.66 ± 0.66	43.51 ± 0.88	63.18 ± 0.29
HB 805	84.99 ± 2.41	28.56 ± 1.03	62.39 ± 0.69	64.61 ± 0.57
HB 806	89.23 ± 0.78	21.45 ± 0.85	51.00 ± 1.98	63.44 ± 0.48
HB 807	91.04 ± 1.43	23.61 ± 0.79	58.88 ± 1.54	66.32 ± 0.93
HB 340	102.68 ± 2.33	11.21 ± 0.92	58.97 ± 1.16	62.55 ± 0.87
av	89.64a	22.50d	54.95a	64.02a

[a] Mean ± standard deviation based on triplicate determinations is reported. Means in the same column with different letters are significantly different ($p \leq 0.05$). [b] Average of sample means in each group.

(2) Acid Hydrolysis. Ground samples (0.1 g) were mixed with 1 mL of 0.2 N H_2SO_4 in test tubes (pH 4.5) and heated for 1 h in a boiling water bath. The acid hydrolysis reaction was terminated by cooling in an ice−water bath for 10 min, and the samples were centrifuged at 10000*g* for 10 min. The supernatant was analyzed by HPLC.

(3) Acid and α-Amylase Hydrolysis. Ground samples were prepared according to the method of Zupfer et al. (*13*) with minor modifications. Ground samples (0.1 g) were mixed with 1 mL of 0.2 N H_2SO_4 in test tubes and heated in a boiling water bath for 1 h. Hydrolysis was terminated by cooling samples (pH 4.5) in an ice−water bath for 10 min prior to the addition of 0.2 mL of 2.5 M aqueous sodium acetate solution containing 2% (w/v) α-amylase. The samples were incubated at 30 °C for 1 h and then centrifuged at 10000*g* for 10 min. The supernatant was analyzed by HPLC.

(4) Acid, α-Amylase, and Cellulase Hydrolysis. The procedure is similar to the acid and amylase hydrolysis procedure up to the centrifugation step. After samples were incubated for 1 h with α-amylase, 0.1 mL of a 0.1 M aqueous sodium acetate solution containing 2% (w/v) cellulase was added, and the samples were incubated at 30 °C for 10 h followed by centrifugation at 10000*g* for 10 min. The supernatant was analyzed by HPLC.

Identification and Quantification of Phenolic Acids by HPLC. Separation and quantitation of phenolic acids in barley were performed using high-performance liquid chromatography, a Varian 5000 HPLC (Varian, Mississauga, ON, Canada), equipped with a Shimadzu SIL-9A autosampler (Shimadzu Corp., Columbia, MD) and a Waters 486 UV detector (Waters, Milford, MA) at 280 nm. Separation was performed with a Supelcosil LC-18, 5 μm, 4.6 mm × 15 cm (Supelco, Oakville, ON, Canada) column. The data were integrated and analyzed using a Shimadzu Class−VP Chromatography Laboratory automated software system (Shimadzu Corp.). The mobile phase utilized a gradient composed of a 0.01 M sodium citrate buffer (A) pH 5.4 adjusted with 50% acetic acid and methanol (B). The best separation was obtained using the following gradient: 0 min, 2% B; 12 min, 4% B; 20 min, 13% B; 22 min, 13% B; 26 min, 2% B. Run time was 30 min. The solvent flow rate was 1.0 mL/min, and separation was performed at room temperature. Each phenolic acid standard (protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, chlorogenic, *p*-coumaric, and ferulic acids) displayed a linear response ($r^2 > 0.95$) over 8−40 μg calibration series. Concentrations of phenolic acids in samples were calculated using syringic acid as an internal standard. Results were expressed in micrograms per gram of dry matter of barley.

Statistical Analysis. Phenolic acid extractions using each treatment were carried out in triplicate, and the mean and standard deviation are reported. Analysis of variance of results was performed using the General Linear Model procedure of SAS Statistical software, version 8 (*17*). Multiple comparisons of the means for each acid content among different groups of barley varieties were carried out by least significance difference (LSD) test at $p \leq 0.05$.

Table 3. Phenolic Acids in Acid and α-Amylase Hydrolytes of Different Barley Varieties[a]

variety	μg/g of dry matter					
	p-hydroxybenzoic acid	vanillic acid	caffeic acid	chlorogenic acid	*p*-coumaric acid	ferulic acid
six-row regular-hulled						
B1602	889.48 ± 0.49	69.23 ± 1.27	5.84 ± 0.23	55.98 ± 0.56	34.38 ± 1.21	222.03 ± 0.33
CDC Sister	722.04 ± 1.24	53.21 ± 0.69	11.98 ± 1.55	88.65 ± 1.92	27.6 ± 0.56	180.94 ± 0.56
Tukwa	727.54 ± 0.77	44.59 ± 1.64	6.59 ± 0.87	70.88 ± 0.36	13.68 ± 0.88	163.62 ± 0.27
AC Lacombe	927.9 ± 0.69	39.28 ± 0.58	9.56 ± 0.91	79.2 ± 1.52	27.61 ± 0.51	238.40 ± 0.91
Kasota	753.71 ± 0.76	59.12 ± 3.21	11.83 ± 0.45	82.64 ± 0.89	18.6 ± 0.65	184.28 ± 0.26
Stander	793.66 ± 1.58	36.9 ± 0.93	12.66 ± 1.25	90.99 ± 0.54	26.14 ± 0.49	209.73 ± 0.54
Robust	793.23 ± 2.13	39.58 ± 0.87	8.83 ± 3.25	104.2 ± 0.87	30.28 ± 0.85	223.28 ± 0.81
Excel	769.49 ± 1.02	36.13 ± 1.32	7.93 ± 1.89	58.63 ± 0.57	26.27 ± 0.57	197.95 ± 0.13
av[b]	797.13d	47.26e	9.40b	78.90d	25.57a	202.53a
two-row regular-hulled						
Harrington	714.08 ± 1.56	53.12 ± 0.58	6.64 ± 0.27	82.24 ± 1.52	9.89 ± 0.36	114.00 ± 0.36
Leduc	829.67 ± 0.52	51.45 ± 1.69	7.31 ± 0.84	65.26 ± 2.10	16.18 ± 0.81	175.66 ± 0.59
Stein	809.11 ± 2.36	49.32 ± 2.56	5.71 ± 0.77	70.12 ± 0.65	10.28 ± 1.29	161.67 ± 0.33
Manley	838.11 ± 0.97	62.15 ± 1.23	7.29 ± 1.86	89.22 ± 0.83	31.22 ± 0.33	194.95 ± 0.51
CDC Dolly	650.85 ± 0.49	55.63 ± 0.89	9.11 ± 1.80	95.9 ± 0.71	27.15 ± 0.85	158.06 ± 0.85
av	768.36e	54.33a	7.21d	80.55c	18.94b	160.87d
six-row regular-hulless						
HB 504	860.24 ± 0.88	53.61 ± 0.77	2.96 ± 0.25	132.26 ± 1.29	14.19 ± 0.59	223.09 ± 0.55
AC Bacon	624.68 ± 1.26	51.54 ± 0.92	11.51 ± 0.55	91.14 ± 0.89	5.73 ± 0.47	182.90 ± 0.43
Falcon	914.52 ± 0.72	44.12 ± 1.66	9.9 ± 0.29	91.52 ± 1.01	1.74 ± 0.26	151.58 ± 0.90
av	799.81c	49.76c	8.12c	104.97a	7.22e	185.86b
two-row regular-hulless						
CDC Richard	1041.99 ± 0.12	49.36 ± 0.82	7.68 ± 0.41	54.21 ± 0.55	2.97 ± 0.12	118.07 ± 0.87
Condor	1061.00 ± 0.59	55.81 ± 0.56	7.91 ± 0.73	58.86 ± 0.63	24.74 ± 0.85	119.93 ± 0.32
HB 313	1001.94 ± 1.23	47.19 ± 0.88	7.57 ± 0.43	66.38 ± 0.99	4.01 ± 0.66	118.82 ± 0.62
HB 801	1080.8 ± 3.25	51.22 ± 0.79	13.66 ± 0.89	61.32 ± 0.21	4.47 ± 1.21	159.08 ± 0.57
HB 803	1180.33 ± 0.87	43.38 ± 1.28	10.38 ± 0.75	68.03 ± 0.45	5.35 ± 0.93	154.46 ± 0.69
SB 89497	1136.9 ± 0.61	55.63 ± 1.39	9.77 ± 0.59	65.41 ± 1.54	3.25 ± 0.26	153.04 ± 0.44
CDC Dawn	799.14 ± 0.52	42.33 ± 0.44	10.83 ± 0.28	110.13 ± 0.92	6.98 ± 0.55	161.45 ± 0.59
HB 335	851.88 ± 0.43	49.63 ± 0.38	9.09 ± 1.27	127.4 ± 1.23	8.63 ± 0.41	172.20 ± 0.23
Phoenix	928.85 ± 1.58	41.65 ± 2.33	5.98 ± 1.11	67.5 ± 0.82	11.52 ± 0.25	106.42 ± 0.81
av	1009.20b	48.47b	9.21d	75.47e	7.99d	140.39e
two-row waxy-hulless						
Candle	1124.68 ± 0.29	49.36 ± 0.66	8.44 ± 0.56	58.35 ± 0.59	1.68 ± 0.11	91.39 ± 0.12
HB 805	851.76 ± 0.73	55.69 ± 0.98	14.46 ± 0.72	112.84 ± 0.32	19.09 ± 0.96	212.17 ± 0.99
HB 806	988.13 ± 1.25	54.28 ± 0.57	11.93 ± 0.98	89.43 ± 0.47	22.02 ± 0.86	221.6 ± 0.93
HB 807	1069.25 ± 1.78	55.68 ± 0.27	10.81 ± 0.85	85.63 ± 0.86	4.83 ± 0.58	195.95 ± 0.98
HB 340	1147.69 ± 3.59	48.79 ± 0.84	10.24 ± 0.67	91.43 ± 0.58	13.6 ± 0.21	198.73 ± 0.82
av	1036.30a	52.76b	11.18a	87.54b	12.24c	183.97c

[a] Mean ± standard deviation based on triplicate determinations is reported. Means in the same column with different letter are significantly different ($p \leq 0.05$). [b] Average of sample means in each group.

RESULTS AND DISCUSSION

Method Development. Previous investigators (*4*, *11*, *12*) have extracted phenolic acids in grain with organic solvents such as methanol, acetone, and hexane following alkaline hydrolysis. This procedure required a longer time for sample preparation. Because of their bifunctional nature, phenolic acids may form both ester and ether linkages through reactions involving their carboxyl and phenolic groups respectively; thus, they participate in the cross-linking of cell wall macromolecules. Those phenolic acids can be released by an acid hydrolysis instead of an alkaline one (results presented later). Others (*7*, *13*) have used single-step hydrolysis treatments involving acid or α-amylase treatments to investigate only the ferulic acid content in barley varieties or cellulase treatments to describe ferulic and *p*-coumaric acids in rice endosperm cell walls. In this study, a sequential treatment of acid plus α-amylase plus cellulase was developed and adopted for sample preparation. Results (presented later) clearly demonstrate that a combined method of acid, α-amylase, and cellulase hydrolysis can release higher amounts of most phenolic acids present in barley. This finding is in accordance with the observation by Shibuya (*7*) that cellulase hydrolyzes 40% of cell wall and releases higher concentrations of ferulic acid.

One of the goals of this work was to develop a rapid method for the analysis of several phenolic acids in barley. The chromatographic separation obtained under our experimental conditions is shown in Figure 1D. A better resolution of all peaks was obtained, and their separation can be achieved in 30 min. In contrast, other researchers were able to obtain separation of the same seven phenolic acids in rye in ∼100 min (*15*).

Hot Water Extraction. Most of the free phenolic acids can be extracted with hot water. Some of the quantities are given in Table 1, and a sample chromatogram of free barley phenolic acids is shown in Figure 1A. The results indicated that protocatechuic acid and chlorogenic acid were the two major compounds that existed in free form in barley grain even though their concentrations were very low (<2.9 μg/g for protocatechuic acid and <16.3 μg/g for chlorogenic acid). Chlorogenic acid content varied depending on the barley variety. No trace of other phenolic acids was found in hot water extracts. Protocatechuic acid was also found in oats and corn at very low levels in free form, 0.5 and 1.1 ppm, respectively (*4*). In the plant, protocatechuic acid was considered to be the oxidation product of either *p*-hydroxybenzoic or *m*-hydroxybenzoic acid and was recognized as a bactericide and fungicide in plant

Table 4. Phenolic Acids in Acid, Amylase, and Cellulase Hydrolysate of Different Barley Varieties[a]

	μg/g of dry matter				
variety	*p*-hydroxybenzoic acid	vanillic acid	chlorogenic acid	*p*-coumaric acid	ferulic acid
six-row regular-hulled					
B1602	919.71 ± 1.56	40.94 ± 0.52	133.28 ± 0.49	37.22 ± 0.86	235.74 ± 0.82
CDC Sister	912.98 ± 0.98	34.17 ± 1.20	191.25 ± 0.76	34.07 ± 1.61	222.93 ± 1.29
Tukwa	1066.4 ± 1.41	35.71 ± 0.59	190.97 ± 0.29	14.45 ± 0.33	228.34 ± 0.96
AC Lacombe	971.65 ± 0.87	29.63 ± 0.92	148.16 ± 0.38	32.01 ± 0.98	220.27 ± 0.55
Kasota	1226.22 ± 0.44	32.15 ± 0.36	197.24 ± 0.85	21.41 ± 0.76	267.29 ± 0.83
Stander	893.98 ± 1.11	37.94 ± 0.67	159.92 ± 1.09	35.1 ± 1.27	208.10 ± 0.91
Robust	958.58 ± 0.92	36.11 ± 0.78	203.41 ± 0.57	34.7 ± 0.55	257.50 ± 0.80
Excel	938.04 ± 0.81	34.83 ± 0.65	124.4 ± 1.32	29.72 ± 0.98	233.48 ± 1.07
av[b]	985.95d	35.19a	168.58c	29.84a	234.21a
two-row regular-hulled					
Harrington	919.01 ± 1.21	35.21 ± 1.12	175.35 ± 0.52	15.19 ± 1.22	283.36 ± 0.61
Leduc	953.34 ± 0.56	32.12 ± 0.86	147.63 ± 0.76	24.48 ± 0.81	289.93 ± 1.46
Stein	976.35 ± 0.67	34.52 ± 0.45	155.63 ± 0.97	24.37 ± 0.99	281.01 ± 0.91
Manley	933.13 ± 0.83	29.66 ± 0.96	145.63 ± 0.45	34.51 ± 1.06	203.73 ± 0.87
CDC Dolly	881.18 ± 0.96	31.29 ± 0.85	172.98 ± 1.01	29.90 ± 0.67	285.99 ± 1.05
av	932.60e	32.56b	159.44d	25.69b	188.80d
six-row regular-hulless					
HB 504	1135.69 ± 0.88	40.12 ± 0.58	193.47 ± 0.62	19.44 ± 0.89	261.81 ± 0.59
AC Bacon	986.78 ± 2.16	37.08 ± 0.76	156.81 ± 0.83	12.99 ± 1.07	249.60 ± 1.29
Falcon	1114.28 ± 0.76	32.13 ± 1.61	204.84 ± 1.41	3.12 ± 1.22	176.26 ± 1.08
av	1078.92c	36.44a	185.04b	11.85d	229.22b
two-row regular-hulless					
CDC Richard	1170.63 ± 0.25	36.62 ± 0.59	178.82 ± 1.92	3.6 ± 1.07	201.94 ± 0.92
Condor	1269.87 ± 0.78	38.12 ± 0.88	174.78 ± 0.87	26.14 ± 0.84	181.98 ± 0.81
HB 313	1138.06 ± 0.51	39.14 ± 0.49	245.85 ± 0.58	11.32 ± 0.80	169.58 ± 0.49
HB 801	1260.44 ± 0.47	42.33 ± 0.21	187.86 ± 1.05	5.97 ± 0.47	206.60 ± 1.08
HB 803	1178.37 ± 1.20	29.78 ± 0.65	154.45 ± 0.69	6.01 ± 0.96	195.19 ± 2.03
SB 89497	1276.32 ± 0.73	36.54 ± 1.96	166.95 ± 0.46	4.57 ± 1.21	182.26 ± 2.11
CDC Dawn	1027.06 ± 0.45	33.15 ± 0.51	175.48 ± 0.78	8.25 ± 0.77	174.23 ± 0.95
HB 335	1044.7 ± 0.29	26.13 ± 0.94	195.14 ± 0.89	9.27 ± 0.62	205.62 ± 0.73
Phoenix	1173.26 ± 0.92	31.56 ± 0.52	184.4 ± 0.73	15.69 ± 0.39	163.95 ± 0.99
av	1170.97a	34.82a	184.86b	10.09e	186.82e
two-row waxy-hulless					
Candle	1158.6 ± 0.46	33.45 ± 1.82	189.61 ± 1.33	4.18 ± 0.73	158.86 ± 1.29
HB 805	1016.9 ± 1.82	36.81 ± 1.32	214.51 ± 1.56	26.33 ± 0.19	249.98 ± 0.96
HB 806	1110.81 ± 0.63	35.16 ± 0.78	182.42 ± 0.57	22.07 ± 0.58	226.68 ± 0.83
HB 807	1132.99 ± 0.84	40.31 ± 0.76	187.79 ± 0.29	6.82 ± 0.73	230.46 ± 1.55
HB 340	1343.38 ± 1.11	39.88 ± 1.03	200.21 ± 0.79	14.29 ± 0.82	206.32 ± 2.09
av	1152.54b	37.12a	194.91a	14.74c	214.46c

[a] Mean ± standard deviation based on triplicate determinations is reported. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Average of sample means in each group.

tissues. The chlorogenic acid was considered to be formed by enzyme-catalyzed *p*-coumaroylquinic acid (*18*).

Acid Hydrolysis. The phenolic acid contents of different barley varieties determined using acid hydrolysis treatment are presented in Table 2, and a sample chromatogram of acid hydrolysate is shown in Figure 1B. From a comparison of parts A and B of Figure 1, it is seen that some phenolic acids can be released by acid hydrolysis. This result confirmed that phenolic acids could esterify with some small molecules of aliphatic alcohols, phenols, phenolic acids, and alkaloids. Among the phenolic acids investigated, the concentrations of protocatechuic, vanillic, chlorogenic, and ferulic acids could be quantified. On the other hand, *p*-hydroxybenzoic, caffeic, and *p*-coumaric acids could be detected but not quantified because of poor resolution or low levels of those compounds. All barley types were significantly different ($p \leq 0.05$) in their vanillic and ferulic acid contents with two-row regular-hulled and two-row waxy-hulless having the highest levels, respectively. Two-row waxy-hulless barley had the highest ($p \leq 0.05$) levels of protocatechuic and ferulic acids. Two-row waxy-hulless barley had the highest ($p \leq 0.05$) levels of chlorogenic acid, and six-row regular-hulled had the lowest one. There was no significant difference ($p > 0.05$) between six-row regular-hulled and six-row regular-hulless in protocatechuic acid content. Some of barley varieties had very high concentrations of protocatechuic acid (SB 89497, 101 μg/g; HB 340, 102 μg/g). The concentration of ferulic acid varied widely among the varieties investigated, ranging from 10 μg/g (Phoenix) to 69 μg/g (HB 335). Ferulic acid is the major low molecular weight phenolic in barley. The genetic or cultivar differences in ferulic acid concentration could result from differences in the caryopsis structure (*13*). For example, Zupfer et al. (*13*) found that smaller grains have a high ferulic acid concentration. In our study, ferulic acid is the main compound that could be hydrolyzed by acid, which indicated that ferulic acid may be in simply esterified form.

Acid and α-Amylase Hydrolysis. The phenolic acid contents of different barley varieties determined using acid plus α-amylase hydrolysis are presented in Table 3, and a sample chromatogram is shown in Figure 1C. After acid hydrolysis with α-amylase hydrolysis, a better separation for all phenolic acids in the complex medium was obtained. All six phenolic acids could be separated except for protocatechuic acid. This result showed that α-amylase was able to break down starch, and the ester linkage between phenolic acids and the attached sugar was hydrolyzed. Barley contains 78−83% (dry weight

basis) of carbohydrates (starch, 63−65%; sucrose, 1−2%; other sugars, 1%; water-soluble polysaccharides, 1−1.5%; alkali-soluble polysaccharides, 8−10%; and cellulose, 4−5%) (*19*). Barley starch can be hydrolyzed by the α-amylase enzyme to form maltose, which is a smaller molecule, and more phenolic acids that were bound to starch were released. Ferulic acid was the major phenolic acid (ranging from 91 μg/g in Candle to 238 μg/g in AC Lacombe). Two-row waxy-hulless barley had the highest ($p \leq 0.05$) *p*-hydroxybenzoic and caffeic acid levels, two-row regular-hulled barley was highest ($p \leq 0.05$) in vanillic acid, and six-row regular-hulless was highest in chlorogenic acid, whereas the highest ($p \leq 0.05$) levels of *p*-coumaric and ferulic acids were found in six-row regular-hulled barley. There was no significant difference ($p > 0.05$) between six-row regular-hulled and two-row regular-hulless in caffeic acid content. Zupfer et al. (*13*) used a similar treatment to investigate ferulic acid in barley and reported substantially higher values (343−580 μg/g) than those found in this study (91−223 μg/g). This difference may be attributed to the effect of genetic and environmental factors.

Acid, α-Amylase, and Cellulase Hydrolysis. The phenolic acid contents of different barley varieties determined using a combination of acid, α-amylase, and cellulase hydrolysis are presented in Table 4. A sample chromatogram is shown in Figure 1D. The cellulase treatment increased the concentrations of *p*-hydroxybenzoic, vanillic, chlorogenic, *p*-coumaric, and ferulic acids. The separation of protocatechuic and caffeic acids was changed strangely. The reason may be that the enzyme is effective in catalyzing protocatechuic or caffeic acid to other compounds.

Plant cell walls consist of a reinforced, multicomponent matrix of noncovalently and covalently cross-linked polymers in which a network of cellulose microfibrils is embedded (*19*). Polysaccharides are the principal constituents of the cell wall. In barley, the major polysaccharide components are arabinoxylan and 1→3,1→4 β-glucan, making up of 71 and 26% of the endosperm cell walls, respectively. After the addition of a degradative enzyme (cellulase), those components were degraded to simple carbohydrate and organic acids, leading to the release of more phenolic acids that were bound to arabinoxylan. The major phenolic acids were *p*-hydroxybenzoic, ferulic, and chlorogenic, vanillic, caffeic, and *p*-coumaric acids. The concentration of *p*-hydroxybenzoic acid was highest followed by ferulic acid. Six-row regular-hulled barley had the highest ($p \leq 0.05$) levels of vanillic acid (similar to six-row regular-hulless, two-row regular-hulless, and two-row waxy-hulless) and *p*-coumaric and ferulic acids, whereas two-row waxy-hulless was highest ($p \leq 0.05$) in *p*-hydroxybenzoic, caffeic, and chlorogenic acids. There was no significant difference ($p > 0.05$) between six-row regular-hulless and two-row regular-hulless in chlorogenic acid content.

Among the three different treatments studied, the treatment combining acid, α-amylase, and cellulase yielded the highest concentration for most phenolic acids, indicating that most phenolic acids in barley are primarily bound to other grain components (for example, starch, cellulose, β-glucan, pentosans, and others). The results were also in accordance with those of Andreasen et al. (*15*), who observed that some commercial cell wall enzyme (Macer, Grindamyl, Rapidase, and Viscozyme) preparations were able to facilitate the release of ferulic acid and other phenolic acids from ground rye grain.

Conclusions. This study demonstrated that phenolic acids existed in barley grain in free and bound forms. The different treatments indicated that phenolic acids were esterified with different components that basically exist in barley. The acid, α-amylase, and cellulase hydrolysis with the developed HPLC protocol will be a more complete method to identify and quantify some of the phenolic acids in barley. *p*-Hydroxybenzoic acid was the major phenolic acid in the 30 barley varieties studied by using acid, α-amylase, and cellulase hydrolysis treatments. Other phenolic acids commonly exist in barley. The concentrations of the various phenolic acids among the different variety groups changed widely. A typical HPLC analysis of phenolic acids takes >30 min, whereas the method described in this study takes only 30 min to identify seven phenolic acids and quantify some of the phenolic acids. This improved HPLC method may be suitable for analyzing phenolic acids in barley and other grains.

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