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Phenolic Acids in Some Cereal Grains and Their Inhibitory Effect on Starch Liquefaction and Saccharification

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ABSTRACT: The presence of phenolic acids in cereal grain is thought to influence starch hydrolysis during liquefaction and saccharification of grain flours in the bioethanol industry. As a basis for remodeling starch hydrolysis systems and understanding inhibition mechanisms, the composition and concentration of phenolic acids in whole grain flours of triticale, wheat, barley, and corn were analyzed by high-performance liquid chromatography. The total phenolic acid contents (sum of nine phenolic acids) in the four grains were 1.14, 1.70, 0.90, and 1.25 mg/g, respectively, with more than 90% found in the bound form. Ferulic, coumaric, and protocatechuic acids were the major phenolic acids in triticale and wheat. Gallic acid was also rich in triticale. Ferulic, coumaric, hydroxybenzoic, and gallic acids were predominant in barley. In corn, ferulic, coumaric, gallic, and syringic acids were abundant. On the basis of these profiles, pure phenolic acids were added individually and collectively to isolated starches at amounts either equivalent to or 3 times those in the whole grains for hydrolysis. The degree of starch hydrolysis with α-amylase and amyloglucosidase decreased up to 8% when individual phenolic acids were present in cooked starch slurry. The decreases were more pronounced when phenolic acids were added collectively (4−5% with α-amylase and 9−13% with sequential α-amylase and amyloglucosidase). The study of a phenolic acid−starch−enzyme model system indicated that the interactions of phenolic acid−enzyme and phenolic acid−starch significantly contributed to the inhibitory effect of starch hydrolysis. Heating facilitated the interactions. Phenolic acids thus play a significant role in the resistance of starch to enzyme and/or the loss of enzyme activity during starch hydrolysis.

KEYWORDS: *Phenolic acids, cereal, starch, enzymatic hydrolysis, HPLC*

INTRODUCTION

Today, the first-generation bioethanol industry using cereal grains as starting material is still the most dominant entity of the bioenergy sector in North America. Cereal grains are rich in starch, which is converted into fermentable sugars through a common liquefaction and saccharification process using α-amylase and amyloglucosidase. However, quantitative conversion of starch from cereal grain is a challenging step that still remains costly and suffers from low conversion efficiency because of incomplete starch hydrolysis at a commercial scale. The presence of non-starch components in grain, such as phenolic compounds, is thought to interfere with starch amylolysis during liquefaction and saccharification of whole grain flours.[1,2]

Cereal grains contain a variety of phenolic compounds, including phenolic acids, flavonoids, tannins, lignans, and other polyphenols, which are distributed non-uniformly throughout grain tissues. They are mainly concentrated in the outer layers of the grains, such as pericarp, testa, and aleurone layers, and considerable amounts also are present in the endosperm and germ of kernels.[3−5] The phenolic compounds are diverse in their concentration, composition, and structure in various grains and have been clearly demonstrated that they are present as both free (e.g., proanthocyanidins or flavonoids) and bound forms (e.g., those are esterified to cell-wall polymers and consist mainly of ferulic acid and its oxidatively coupled dimers), but the majority is bound to the insoluble portion of fiber.[4−7] Ferulic acid is the most abundant phenolic acid in common cereals, representing up to 90% of total phenolic compounds, and other phenolic acids, such as vanillic, syringic, *p*-coumaric, caffeic, and *p*-hydroxybenzoic acids, are present in considerably lower amounts in triticale, wheat, rye, oat, and corn grains.[5,8−11]

Numerous references in the literature point to the inhibitory effect of phenolic compounds on enzymatic starch hydrolysis by α-amylase and amyloglucosidase, an effect which is generally attributed to the ability of polyphenols to decrease enzyme activity by binding enzymes/proteins.[1,12−16] However, phenolic acids with their carboxyl and hydroxyl groups are also capable of binding with starch and other polysaccharides through hydrogen bonds, chelation, or covalent bonds, forming bridges or cross-links.[17] The effect of tannic acid and catechin on legume starch hydrolysis[18] as well as the interference of gallic and chlorogenic acids with the starch−iodine reaction[19] have been reported. However, a specific interaction of phenolic acid with starch and its inhibitory effect on starch hydrolysis have not been reported.

This study was designed to investigate the effects of phenolic acids either alone or in combination on the hydrolysis of starches from triticale, wheat, barley, and corn grains using standard phenolic acids (purchased commercially) to determine the nature of those effects and whether they are influenced by incubation temperature. The addition level of phenolic acids was comparable to that in the native grains as calculated on the basis of the compositional analysis of grain meals for phenolic acids. The outcome of the research is expected to provide a

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better understanding of the inhibitory mechanisms of grain phenolic compounds on starch amylolysis (liquefaction and saccharification) for ethanol production, thus leading to define strategies (i.e., preprocessing and breeding efforts) for improved starch conversion.

MATERIALS AND METHODS

Materials. Cereal grains of triticale, barley, wheat, and corn used in this study were the same as our previous report.[2] Barley (*Hordeum vulgare* L. cv. Xena) was obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. Corn (*Zea mays* L.) was supplied by Pioneer Hybrid, Ltd., Chatham, Ontario, Canada. Canada Prairie Spring (CPS) wheat (*Triticum aestivum* L.) was provided by Alberta Agriculture and Food, Barrhead, Alberta, Canada. Triticale (x *Triticosecale* cv. Pronghorn) was supplied by the Field Crop Development Centre, Alberta Agriculture and Rural Development, Lacombe, Alberta, Canada. The grains (1 kg) were ground in a Retsch mill (model ZM 100, Haan, Germany) using a ring sieve with an aperture size of 0.5 mm. Ground flours were stored in Fisherbrand polyprophlene 32 oz jars at 5 °C. Liquozyme SC (α-amylase) (120 KNU/g) and Spirizyme Fuel (amyloglucosidase) (750 AGU/g) enzymes were kindly provided by Novozymes, Bagavaerd, Denmark. Pure starches were isolated from ground grain flours (0.5 kg) using laboratory wet-extraction procedures of Mistry and Eckhoff,[20] Vasanthan and Temelli,[21] and Wolf[22] for wheat and triticale, corn, and barley, respectively. In brief, the isolation involved preparing a dough or slurry with deionized water followed by dilute alkali washing to separate the protein-enriched fiber from the starch milk. The starch milk was then subjected to centrifugation and water washing to obtain pure starch (>94%).

High-performance liquid chromatography (HPLC)-grade phenolic acid standards (*p*-coumaric, ferulic, *p*-hydroxybenzoic, vanillic, caffeic, chlorogenic, protocatechuic, gallic, and syringic acids) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All other reagents used were of analytical and HPLC grade.

Determination of Phenolic Acids Using HPLC. Free phenolic acids in grains were extracted by mixing 1 g of the ground flour sample with 20 mL of 50% methanol for 1 h. After centrifugation at 4000*g* for 5 min, the supernatants were collected and used for HPLC analysis. Bound phenolic acids were extracted according to the method by Yu et al.[11] Ground samples (1 g) were mixed with 10 mL of 0.2 N H_2SO_4 in test tubes and heated in a boiling water bath for 1 h to release bound phenolic acids. Hydrolysis was terminated by cooling samples in an ice−water bath for 10 min, and the pH was adjusted to 4.5 using 0.2 N NaOH prior to the addition of 2 mL of 2.5 M aqueous sodium acetate solution containing 8% (w/v) thermostable α-amylase. The samples were incubated in boiling water for 1 h and then centrifuged at 4000*g* for 10 min. The supernatant was analyzed by HPLC for individual phenolic acids (free and bound).

HPLC analyses were performed using an Agilent pump (Agilent Technologies 1200 series, Palo Alto, CA) equipped with an Agilent 717 plus autosampler coupled with a Agilent diode array detector (DAD) at 360, 280, and 254 nm. Separation was performed with a Zobax 300 SB-C18 (5 μm, 4.6−250 mm) column (Agilent, Palo Alto, CA) at room temperature. Elution was carried out using a gradient procedure with a mobile phase containing solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) as follows: 0 min, 5% B; 15 min, 20% B; 35 min, 40% B; 42 min, 65% B; 50 min, 80% B; 52 min, 5% B; and 60 min, 5% B. The run time was 60 min; the solvent flow rate was 1.0 mL/min; and the injection volume was 10 μL. Agilent "Chemstation" software, version 2007, has been used for calculations of phenolic acids. The concentrations of individual phenolic acids were calculated using standard curves. Results were expressed in micrograms per gram.[23]

Chemical Composition. The moisture content was determined by the standard procedure of AACC (method 44-15A).[24] The total starch content was estimated according to the total starch assay of Megazyme International, Ireland, Ltd. (Wicklow, Ireland).

Starch Amylolysis in the Presence of an Individual or a Mixture of Phenolic Acids. The effect of phenolic acids on starch amylolysis was conducted by two experiments. One experiment was performed by adding individual pure phenolic acids (in "free" form) to starch slurries at amounts either equivalent to or 3 times those in the whole grain flours (free and bound). The second experiment was performed by adding a mixture of the major phenolic acids to starch slurries at amounts equivalent to those in the whole grain flours. The protocols for starch amylolysis using α-amylase or sequential α-amylase and amyloglucosidase are shown in Figure 1.

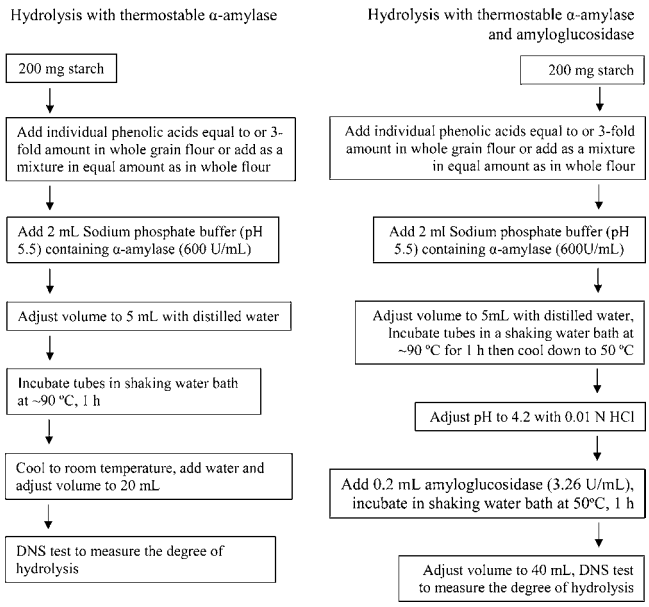

Figure 1. Protocols for starch amylolysis in the presence of phenolic acids.

Model Reaction System for the Interaction of Phenolic Acid−Starch−Enzyme. To study the interactions of phenolic acids with amylolytic enzymes and starch, a model reaction system was designed with four sets of solutions/slurries, which included pure "free" chemical phenolic acid, isolated starch (4%, w/v), and/or α-amylase (150 units) in deionized water (Figure 2). Ferulic acid and coumaric acid were chosen for this study because they are the two most abundant phenolic acids in cereal grains. The phenolic acid concentration was equal to the level of total ferulic acid or coumaric acid in whole grain flour. Boiling was performed to simulate the liquefaction step as in the bioethanol process. As shown in Figure 2,

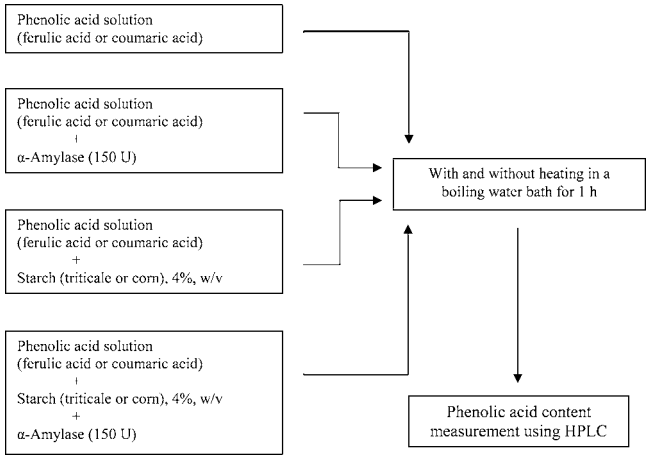

Figure 2. Phenolic acid−starch−enzyme model system.

the first set of solution, ferulic or coumaric acid solution, was prepared as a control. The second set of phenolic acid solution included α-amylase (150 units). In the third set of phenolic acid solution, isolated triticale or corn starch [4%, w/v, with a starch purity of 97.9 and 93.8%, dry basis (db), respectively] was added to the solution. For the fourth set of phenolic acid solution, triticale or corn starch (4%, w/v) and α-amylase (150 units) were added. Four sets of solutions were treated with heating in a boiling water bath under shaking or without heating. The contents of ferulic or coumaric acid were then analyzed by HPLC according the method described by Zhao et al.[23]

Determination of the Degree of Starch Hydrolysis. The concentration of reducing sugars in the supernatant of samples after centrifugation was determined by the dinitrosalicylic acid (DNS) method,[25] and the degree of hydrolysis was expressed as the weight of glucose equivalents per 100 g of dry starch (%, db).

Statistical Analysis. All chemical analyses and experiments were carried out in duplicate at least. Analysis of variance was performed using the general linear model (GLM) procedure of SAS Statistical Software, version 9.1.2 (SAS Institute, Inc., Cary, NC). Multiple comparisons of the means were performed using Tukey's test ($p < 0.05$).

RESULTS AND DISCUSSION

Concentrations of Phenolic Acids in Cereal Grains. The concentrations of nine common phenolic acids in four cereal grains quantified by HPLC are shown in Table 1. The majority of phenolic acids were found in the bound form (90.3% in triticale, 90.3% in wheat, 90.6% in corn, and 91.4% in barley). Among individual phenolic acids identified, the main phenolic acids (content higher than 100 μg/g) were ferulic acid, *p*-coumaric acid, protocatechuic acid, and gallic acid in triticale; ferulic acid, protocatechuic acid, and *p*-coumaric acid in wheat; *p*-hydroxybenzoic acid, gallic acid, *p*-coumaric acid, and ferulic acid in barley; and *p*-coumaric acid, ferulic acid, gallic acid, and syringic acid in corn. Chlorogenic acid was not detectable in triticale and wheat flour. The data indicated that the composition and concentration of phenolic acids in cereal grains vary with grain species and cultivar.

Individual and total phenolic acid contents have been reported in a wide range for various cereal grains in the literature, and those values are difficult to compare because of the diversity of grain varieties, range of environmental factors, and different analytical and extraction methods used.[5,26−29] In comparison to alkaline hydrolysis, which is a reliable method to extract hydroxycinnamic acids, acid hydrolysis allows for efficient extraction of more common phenols.[6] Hydrolysis with sulfuric acid instead of hydrochloric acid improves the release of phenolic compounds bound to cell walls (e.g., polysaccharides and proteins).[29] Enzymatic hydrolysis (e.g., α-amylase, cellulase, and other cell-wall-degrading enzymes) is able to release bound phenolic compounds in cell walls.[11,30] In this study, the combination of acid hydrolysis and enzymatic degradation, as reported by Yu et al.,[11] was possibly applicable to quantify more accurately the total phenolic acids in cereal grains. The release of phenolic acids from bound to free form through α-amylase hydrolysis also is meanful for starch liquefaction because of similar hydrolysis conditions.

Effect of Individual Phenolic Acids on Starch Hydrolysis with α-Amylase and Sequential α-Amylase and Amyloglucosidase. The addition of individual "free" phenolic acids to starch slurries in amounts of individual phenolic acid equivalents to whole flours resulted in a slight decrease in the degree of hydrolysis of starch with α-amylase alone (up to 5%) (Table 2) and both α-amylase and amyloglucosidase (up to 6%) (Table 3) in all four isolated

Table 1. Phenolic Acids in Whole Grain Flours (μg/g, db)

sample	*p*-coumaric acid	ferulic acid	*p*-hydroxybenzoic acid	vanillic acid	caffeic acid	chlorogenic acid	protocatechuic acid	gallic acid	syringic acid	total phenolics
triticale										
free	ND[a]	20.3 ± 0.2	ND	ND	4.8 ± 0.2	ND	72.7 ± 0.9	ND	12.6 ± 0.2	110.3
bound[b]	258.5	463.4	7.4	15.4	9.2	ND	126.5	123.4	21.6	1025.2
total	258.5 ± 0.3	483.7 ± 1.3	7.4 ± 0.2	15.4 ± 0.1	13.9 ± 0.1	ND	199.1 ± 1.2	123.4 ± 0.8	34.1 ± 0.7	1135.5
wheat										
free	ND	5.4 ± 0.6	ND	ND	11.2 ± 0.4	ND	141.1 ± 0.8	ND	7.6 ± 0.3	165.3
bound	293.0	766.2	9.2	19.5	40.7	ND	313.2	66.1	26.9	1534.8
total	293.0 ± 0.4	771.6 ± 0.5	9.2 ± 0.0	19.5 ± 0.3	51.9 ± 0.5	ND	454.3 ± 1.4	66.1 ± 0.2	34.5 ± 0.2	1700.1
barley										
free	5.0 ± 0.6	ND	ND	9.8 ± 0.1	ND	34.2 ± 0.1	29.1 ± 0.2	ND	ND	78.1
bound	146.4	132.1	215.0	39.5	21.9	42.9	36.8	158.6	32.2	825.4
total	151.4 ± 1.1	132.1 ± 0.6	215.0 ± 0.4	49.3 ± 0.3	21.9 ± 0.0	77.1 ± 1.1	65.9 ± 0.1	158.6 ± 1.0	32.2 ± 0.4	903.5
corn										
free	15.5 ± 0.4	37.5 ± 0.4	ND	5.2 ± 0.3	ND	17.9 ± 0.4	20.1 ± 0.2	ND	21.5 ± 0.7	117.7
bound	568.5	228.1	11.6	10.2	24.4	60.0	27.0	116.5	86.8	1133.0
total	584.0 ± 1.0	265.5 ± 0.4	11.6 ± 0.3	15.4 ± 0.7	24.4 ± 1.5	77.9 ± 0.7	47.0 ± 0.3	116.5 ± 1.0	108.4 ± 0.0	1250.7

[a]ND = not detectable. [b]Bound phenolic acids were calculated by the difference of total and free phenolic acids.

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Table 2. Degree of Hydrolysis (%, db) of Starches Treated by α-Amylase in the Presence of Individual Phenolic Acids[a]

	triticale		wheat		barley		corn	
phenolic acid	X1	X3	X1	X3	X1	X3	X1	X3
control	44.3 ± 0.4 a	44.3 ± 0.4 a	45.3 ± 0.1 a	45.3 ± 0.1 a	45.1 ± 0.2 a	45.1 ± 0.2 a	43.2 ± 0.3 a	43.2 ± 0.3 a
p-coumaric acid	41.2 ± 0.6 e	39.6 ± 0.1 e	41.5 ± 0.3 d	39.2 ± 0.3 g	43.2 ± 0.3 bc	42.0 ± 0.0 c	38.2 ± 0.4 e	37.4 ± 0.6 d
ferulic acid	42.1 ± 0.4 cd	39.7 ± 0.1 e	42.8 ± 0.6 c	40.3 ± 0.4 ef	43.2 ± 0.1 bc	42.2 ± 0.1 c	40.9 ± 0.2 bcd	39.9 ± 0.4 bc
p-hydroxybenzoic acid	42.8 ± 0.3 c	38.4 ± 0.1 f	42.9 ± 0.3 c	41.0 ± 0.1 e	42.6 ± 0.9 c	41.0 ± 0.3 d	42.3 ± 0.1 ab	39.6 ± 0.8 bcd
vanillic acid	42.7 ± 0.2 c	40.7 ± 0.5 d	42.7 ± 0.3 cd	40.6 ± 0.2 e	43.3 ± 0.3 bc	41.7 ± 0.1 c	41.9 ± 0.4 bcd	38.0 ± 0.5 cd
caffeic acid	41.6 ± 0.2 de	40.8 ± 0.3 d	43.2 ± 1.0 bc	42.0 ± 0.4 d	43.7 ± 0.1 bc	41.7 ± 0.3 c	40.7 ± 0.6 cd	38.0 ± 0.7 cd
chlorogenic acid					43.4 ± 0.3 bc	41.8 ± 0.2 c	41.0 ± 0.3 bcd	38.7 ± 0.7 bcd
protocatechuic acid	41.0 ± 0.3 e	39.5 ± 0.2 e	42.9 ± 0.3 c	39.7 ± 0.6 fg	43.4 ± 0.1 bc	40.7 ± 0.2 d	40.4 ± 0.4 d	39.7 ± 0.8 bcd
gallic acid	42.6 ± 0.1 c	41.6 ± 0.4 c	43.5 ± 0.3 bc	42.4 ± 0.1 cd	43.1 ± 0.3 bc	41.0 ± 0.3 d	41.7 ± 0.8 bcd	40.3 ± 1.1 b
syringic acid	43.4 ± 0.0 ab	43.9 ± 0.4 b	44.4 ± 0.8 ab	43.0 ± 0.4 c	43.1 ± 0.2 bc	42.0 ± 0.1 c	42.0 ± 0.3 bc	41.0 ± 0.2 b

[a]Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$). X1 and X3 represent the addition level of phenolic acid as equal and 3-fold the amount in whole grain flour, respectively.

Table 3. Degree of Hydrolysis (%, db) of Starches Treated by α-Amylase and Amyloglucosidase in the Presence of Individual Phenolic Acids[a]

	triticale		wheat		barley		corn	
phenolic acid	X1	X3	X1	X3	X1	X3	X1	X3
control	86.1 ± 0.4 a	86.1 ± 0.4 a	87.5 ± 0.3 a	87.5 ± 0.3 a	90.1 ± 0.1 a	90.1 ± 1.1 a	82.8 ± 0.5 a	82.8 ± 0.5 a
p-coumaric acid	83.3 ± 0.1 c	79.3 ± 0.1 de	83.8 ± 0.1 c	79.5 ± 0.6 e	86.1 ± 0.6 bc	83.6 ± 0.8 cd	79.5 ± 0.7 bc	77.4 ± 0.4 c
ferulic acid	82.2 ± 0.4 cd	80.0 ± 0.4 d	84.0 ± 0.6 c	81.8 ± 0.3 d	86.5 ± 0.8 bc	84.7 ± 0.1 bcd	78.8 ± 0.4 bc	75.8 ± 0.2 de
p-hydroxybenzoic acid	80.2 ± 0.4 f	78.6 ± 0.4 e	81.8 ± 0.6 d	80.4 ± 0.7 e	87.4 ± 1.0 b	85.0 ± 0.6 bc	77.9 ± 0.2 cd	75.5 ± 0.1 de
vanillic acid	82.8 ± 0.3 cd	81.6 ± 0.4 bc	83.8 ± 0.3 c	81.6 ± 0.3 d	85.1 ± 0.6 c	83.9 ± 0.4 cd	78.3 ± 0.3 bc	75.0 ± 0.3 e
caffeic acid	82.8 ± 0.8 cd	81.2 ± 0.9 c	84.1 ± 0.4 c	81.9 ± 0.1 cd	86.1 ± 0.7 bc	85.0 ± 0.8 bc	77.8 ± 0.4 cd	75.9 ± 0.2 de
chlorogenic acid					85.1 ± 0.6 c	83.6 ± 0.8 cd	76.8 ± 0.6 d	75.0 ± 0.2 e
protocatechuic acid	84.7 ± 0.3 b	82.3 ± 0.2 bc	85.8 ± 0.2 b	83.3 ± 0.3 b	86.3 ± 1.0 bc	86.0 ± 0.6 b	79.1 ± 0.5 bc	77.5 ± 0.3 c
gallic acid	81.9 ± 0.4 de	79.4 ± 0.6 de	83.7 ± 1.3 c	81.4 ± 0.2 d	85.5 ± 1.0 c	83.3 ± 0.6 d	78.8 ± 0.7 bc	76.2 ± 0.4 d
syringic acid	81.9 ± 0.6 de	80.0 ± 0.2 d	84.2 ± 0.1 c	82.9 ± 0.7 bc	86.0 ± 0.3 bc	83.6 ± 0.7 cd	79.9 ± 0.2 b	78.6 ± 0.2 b

[a]Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$). X1 and X3 represent the addition level of phenolic acid as equal and 3-fold the amount in whole grain flour, respectively.

Table 4. Degree of Hydrolysis (%, db) of Starches Treated with α-Amylase and Amyloglucosidase in the Absence and Presence of Phenolic Acids[a]

	α-amylase		α-amylase + amyloglucosidase	
starch source	starch	starch + PAs[b]	starch	starch + PAs
triticale	44.3 ± 0.8 a	40.2 ± 0.3 b	86.1 ± 1.0 c	75.4 ± 0.6 d
wheat	45.3 ± 0.7 a	39.8 ± 0.3 b	87.5 ± 0.5 c	74.3 ± 0.9 d
barley	45.1 ± 0.4 a	41.2 ± 0.8 b	90.1 ± 0.8 c	77.1 ± 0.2 d
corn	43.2 ± 0.3 a	42.7 ± 0.6 b	82.8 ± 0.8 c	73.5 ± 0.5 d

[a]In triticale starch, *p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, and syringic acid; in wheat starch, *p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, naringin, syringic acid, and caffeic acid; in barley starch, *p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, syringic acid, vanillic acid, and chlorogenic acid; and in corn starch, *p*-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, syringic acid, and chlorogenic acid. Means in the same row with different letters are significantly different at $p < 0.05$. [b]Phenolic acids (PAs) added to starch slurry in the equal amounts as in whole grains.

starches. The degree of hydrolysis further decreased when 3-fold amounts of individual phenolic acids were added (up to 6% with α-amylase and up to 8% with both α-amylase and amyloglucosidase). The study indicated that an inhibitory effect of phenolic acids on starch amylolysis occurred; however, the inhibition was limited because of the low individual phenolic acid concentration. There was no obvious trend in the effect of each phenolic acid on starch hydrolysis. In contrast, Rohn et al.[13] reported that the inhibitory effect of phenolic acids on α-amylase activity follows the order *p*-benzoquinone > chlorogenic acid > gallic acid > caffeic acid > ferulic acid > quinic acid, depending upon the concentration and the number and position of hydroxyl groups of the phenolic compounds applied.

Effect of the Combination of Major Phenolic Acids on Starch Hydrolysis with α-Amylase and Sequential α-Amylase and Amyloglucosidase. When a combination of major phenolic acids were added to starch slurries at amounts equivalent to whole grain flours, the degree of hydrolysis of starches was significantly ($p < 0.05$) decreased in all starches (4−5% with α-amylase and 9−13% with sequential α-amylase and amyloglucosidase) (Table 4). In comparison to the starch hydrolysis with α-amylase and amyloglucosidase alone, the hydrolysis with sequential α-amylase and amyloglucosidase showed a higher decrease in the degree of hydrolysis by 11.2, 12.4, 14.4, and 15.1% for corn, triticale, barley, and wheat starches, respectively, indicating a combinational effect of

Table 5. Phenolic Acid Contents in the Reaction Mixture of Phenolic Acid, Starch, and Enzyme with or without Boiling Treatment[a]

	triticale		corn	
treatment	coumaric acid (μg)	ferulic acid (μg)	coumaric acid (μg)	ferulic acid (μg)
phenolic acid	191.9 ± 0.7 a	357.6 ± 0.4 a	374.5 ± 0.2 a	172.0 ± 0.6 a
phenolic acid + boiling	192.3 ± 0.2 a	357.7 ± 0.3 a	376.8 ± 0.7 a	178.8 ± 0.7 ab
enzyme + phenolic acid	185.2 ± 0.2 b	351.4 ± 0.6 b	364.0 ± 1.5 b	167.2 ± 1.0 ab
enzyme + phenolic acid + boiling	142.0 ± 0.9 f	287.0 ± 0.8 f	285.8 ± 1.4 e	129.9 ± 0.5 d
starch + phenolic acid	175.1 ± 0.2 d	345.5 ± 1.5 d	374.5 ± 1.0 a	169.6 ± 1.0 ab
starch + phenolic acid + boiling	168.5 ± 0.9 e	317.4 ± 0.8 e	341.1 ± 0.7 d	126.5 ± 0.9 d
starch + enzyme + phenolic acid	179.9 ± 0.6 c	348.1 ± 0.8 c	352.7 ± 1.1 c	160.4 ± 0.9 c
starch + enzyme + phenolic acid + boiling	130.2 ± 0.9 g	284.2 ± 1.0 g	272.6 ± 0.4 f	117.6 ± 0.4 e

[a]Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$).

phenolic acids on starch hydrolysis when a mixture of phenolic acids was present.

A number of studies have indicated that the inhibitory effect of phenolic compounds on α-amylase and amyloglucosidase activities is concentration-dependent.[12,15,16] The type and structure of phenolic compounds, such as the number and position of hydroxyl groups, play a significant role.[12,13,31,32] Phenolic acids may bind to the active site of enzymes or to the secondary binding site of enzyme−substrate complexes in an uncompetitive inhibition mode.[33]

Interaction of Phenolic Acids with Starch and Enzyme during Amylolysis. Two of the major phenolic acids, coumaric and ferulic acids, were used to study the interactions of phenolic acid with triticale and corn starches and/or α-amylase. As shown in Table 5, the concentration of coumaric and ferulic acids in solution was not significantly ($p < 0.05$) changed with and without boiling. When α-amylase was added to the phenolic acid solution and mixed for 60 min, the contents of both coumaric and ferulic acids were slightly reduced (up to 3%) without boiling and significantly ($p < 0.05$) reduced (20−26%) with boiling, indicating that thermal treatment greatly induced the interaction of phenolic acids with enzyme (α-amylase and amyloglucosidase). A significant reduction ($p < 0.05$) of the phenolic acid concentration was observed in the phenolic acid−triticale starch mixture (9% in coumaric acid and 4% in ferulic acid, respectively) but not in the phenolic acid−corn starch mixture. When the mixture of phenolic acid−starch was boiled, a significant ($p < 0.05$) decrease in the phenolic acid concentration occurred in both mixtures (9−26%). When both starch and enzyme were added to the phenolic acid solution, the phenolic acid contents were reduced by up to 7% compared to the phenolic acid solution alone and the enzyme−phenolic acid mixture. Boiling resulted in a further decrease of the contents of both coumaric or ferulic acid in the starch−enzyme−phenolic acid mixture (18−28%). Thus, the total loss of phenolic acid in the starch−enzyme−phenolic acid system with boiling was 21−32%. The study clearly indicated that interactions occurred between phenolic acid and enzyme and between phenolic acid and starch, depending upon the starch source, phenolic acid strucutre, and reaction conditions (e.g., heating temperature and time). Boiling played a significant role in facilitating the interaction between phenolic acid and starch and enzyme. Both amylose and amylopectin molecules in starch granules may interact with phenolic compounds through forming inclusion complexes with amylose molecules[34] and by binding to side chains of amylopectin and the amorphous region of starch granules, thus altering starch physicochemical properties.[35,36] The free phenolic acids in grain flour and released phenolic acids during hydrolysis may bind to the starch chains, increasing the resistance of starch to further enzymatic hydrolysis, especially during starch liquefaction using thermostable α-amylase at high temperature (e.g., boiling). The inhibitory effect of phenolic compounds on the conversion of starch to ethanol lies mainly in the inhibition of amylase hydrolysis and not in fermentation.[1]

In conclusion, the composition and concentration of phenolic acids in cereal grains varied with grain variety. The presence of phenolic acids in grains inhibited starch hydrolysis. The inhibition of starch hydrolysis was more pronounced in the presence of a mixture of phenolic acids compared to individual phenolic acids. A model system study demonstrated that the interaction between phenolic acid and enzyme as well as between phenolic acid and starch significantly contributed to the inhibitory effect. Boiling enhanced the interactions in the starch−enzyme−phenolic acid system. In light of the above information, we predict that jet cooking of whole grain flours in the presence of thermostable α-amylase may compromise the conversion efficiency of starch into yeast-fermentable sugars. Therefore, in this perspective, cold starch hydrolysis with simultaneous saccharification fermentation (SSF) may be a better approach than jet cooking with SSF. Furthermore, the findings on the interaction of phenolic acids with both enzyme and starch are definitely useful to grain breeders for the selection of grain varieties that are low in phenolics and to bioethanol producers for process optimization (e.g., milling, pearling, pretreatments, jet cooking, etc.).

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Notes

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