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Effects of Solid-State Enzymatic Treatments on the Antioxidant Properties of Wheat Bran

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This study evaluated the potential of solid-state enzyme treatments to release insoluble bound antioxidants such as phenolic acids from wheat bran, thereby improving its extractable and potentially bioaccessible antioxidant properties including scavenging capacities against peroxyl (ORAC), ABTS cation, DPPH and hydroxyl radicals, total phenolic contents, and phenolic acid compositions. Investigated enzyme preparations included Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, Celluclast 1.5L, and porcine liver esterase. Results showed significant dose-dependent increases in extractable antioxidant properties for some enzyme preparations, and Ultraflo L was found to be the most efficient enzyme, able to convert as much as 50% of the insoluble bound ferulic acid in wheat bran to the soluble free form. The effect of moisture content on these solid-state enzyme reactions was also evaluated and found to be dependent on enzyme concentration. These data suggest that solid-state enzyme treatments of wheat bran may be a commercially viable post-harvest procedure for improving the bioaccessibility of wheat antioxidants.

KEYWORDS: Wheat; bran; enzyme; antioxidant; solid-state; phenolic; bioaccessibility; bioavailability

INTRODUCTION

Increasing evidence supporting the role of bioactive food components in preventing and managing health conditions has promoted research to improve the production of bioactives in food crops and their bioavailability in humans. The consumption of foods rich in antioxidants, one type of bioactive food component, has been linked through epidemiological studies to reduced incidences of chronic diseases such as cancer, heart disease, and diabetes (*1*, *2*). Antioxidants are believed to prevent chronic diseases by preventing oxidative damage to important biomolecules such as DNA, membrane lipids, and proteins through multiple mechanisms (*1*−*4*). These mechanisms may include, but are not limited to, quenching free radicals, chelating transition metals, or stimulating antioxidative enzyme systems (*1*−*4*).

Wheat is an important dietary staple and has been found in numerous studies to contain significant antioxidant properties in vitro such as chelating activities against Fe^{2+} and Cu^{2+}, inhibition of low-density lipoprotein and DNA oxidation, and scavenging activities against peroxyl, hydroxyl, 2,2-diphenyl-1-picryhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation, and superoxide anion radicals (*5*−*25*). Phenolic acids present in wheat are thought to significantly contribute to these antioxidant properties and the health benefits of whole grain consumption observed in numerous epidemiological studies (*26*, *27*). Significant levels of phenolic acids, predominately ferulic acid, have been detected in both hard and soft wheat grains (*6*, *11*, *13*, *16*, *21*, *28*) and are found mostly concentrated in the bran fraction (*12*, *29*−*31*). Although some of these phenolic acids exist in free or soluble conjugated forms, the majority are in an insoluble bound form, esterified to plant cell wall material (*6*, *10*, *16*, *24*, *32*−*34*).

The proposed health benefits of phenolic acids and their significant levels in wheat bran have made their bioavailability in humans of recent interest (*32*). Since only free and some conjugated phenolic acids are thought to be available for absorption (bioaccessible) in the human small or large intestines, human absorption of the predominately bound phenolic acids in wheat bran has been shown to be minimal (*35*). It is widely accepted that free phenolic acids are absorbed in the small intestine while bound phenolics could be minimally absorbed in the colon after hydrolysis from the polysaccharide matrix by colonic microflora (*32*, *35*). Given this, the release of bound wheat bran phenolic acids in wheat bran prior to consumption through post-harvesting procedures could be a strategy to improve their bioaccessibility in humans.

The enzymatic hydrolysis of bound phenolic acids from cell wall materials including wheat bran has been previously investigated to produce a natural source of ferulic acid for flavor or pharmaceutical application (*34*). The enzymatic approach has been shown to be effective using xylanases, β-gluconases, and cellulases to break up wheat bran cell wall material combined with enzymes specifically to hydrolyze the ester linked phenolic acids such as cinnamoyl or feruloyl esterases (*34*, *36*−*43*). These studies, however, conducted the enzyme reactions in aqueous systems, which may prove impractical for commercial scale

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Table 1. Summary of Enzyme Preparation Characteristics[a]

enzyme preparation	enzyme class	declared major activity	reported side activities	source
Viscozyme L	β-glucanase (endo-1,3(4)-)	100 FBG/g	xylanase,[b] hemicellulase,[b] cellulase,[b] feruloyl esterase[c]	*Aspergillus aculeatus*
porcine liver esterase	carboxylic esterase	1667 esterase units/mL		porcine liver
Pectinex 3XL	polygalacturonase	3000 PECTU/mL	pectinesterase,[b] hemicellulase,[b] cellulase,[b] xylanase[d]	*Aspergillus aculeatus* and *Aspergillus niger*
Ultraflo L	β-glucanase (endo-1,3(4)-)	45 FBG/g	arabanase,[b] cellulase,[b] pentosanase,[b] xylanase,[b] feruloyl esterase[c]	*Humicola insolens*
Flavourzyme 500L	aminopeptidase	500 LAPU/g		*Aspergillus oryzae*
Celluclast 1.5L	cellulase	700 EGU/g		*Trichoderma reesei*

[a] EGU stands for endo glucanase units; LAPU stands for leucine aminopeptidase units; FBG stands for fungal β-glucanase units; PECTU stands for pectinase units. [b] Identified by manufacturer [c] See ref *42*. [d] See ref *59*.

post-harvest treatments for food ingredients. Solid-state fermentation, however, is widely used commercially to produce foods such as tempeh, miso, and soy sauce (*44*) and to increase the phenolic potential of soybean powder (*45*). The biochemical nature of fermentation is enzyme-catalyzed reactions. Solid-state enzymatic procedures have been developed and used to improve the physiochemical and functional properties of psyllium (*9*), to hydrolyze chestnut starch (*46*), and to enhance the release of phenolics from cranberry pomace (*47*). Solid-state enzymatic reaction systems are practical for food ingredient production because they require no expensive equipment, are environmentally friendly, and do not require postreaction processing to recover products.

Post-harvest solid-state enzymatic procedures have the potential to release insoluble bound phenolic acids from wheat bran and thereby improve their bioaccessibility and potential bioavailability. The objective of this study was therefore to evaluate five selected commercially available food-grade enzyme preparations and one purified enzyme for their potential to improve the extractable free phenolic contents and antioxidant properties of wheat bran through solid-state enzymatic reactions. In addition, this study examined the effects of enzyme to substrate ratios and reaction moisture contents on these extractable phenolic contents and antioxidant properties of wheat bran. No study to date has investigated this opportunity.

MATERIALS AND METHODS

Chemicals and Reagents. 2,2′-Bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and $FeCl_3$ were purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). β-Cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary). Viscozyme L, Pectinex 3XL, Flavorzyme 500L, and Celluclast 1.5L, all enzyme preparation products produced by Novozymes Corp. (Bagsvaerd, Denmark), were purchased from Sigma-Aldrich with product numbers of V2010, P2736, P6100, and C2730. Porcine liver esterase was purchased from Sigma-Aldrich (E2884). Ultraflo L enzyme preparation was a gift from Novozymes North America (Franklinton, NC). Details of all enzyme properties are listed in Table 1. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Hard Winter Wheat Bran Samples. Bran from Akron and Jagalene wheat varieties, both commonly produced red winter wheat varieties, were provided by Dr. Scott Haley in the Department of Soil and Crop Science, Colorado State University, Fort Collins, CO 80523. Both varieties were grown in Fort Collins, Colorado, during the 2004 growing season under agronomic practices considered typical for wheat production in eastern Colorado. Harvested grain samples from each location were cleaned using seed cleaners to remove all non-grain debris present and stored under ambient temperature. Grain samples were ground and separated into flour and bran using a Brabender Quadromat Junior experimental mill.

Solid-State Enzymatic Reactions. Bran samples were ground to 40-mesh using a micro-mill manufactured by Bel Art Products (Pequannock, NJ) and tempered to a moisture content of 10%. The individual enzyme preparations were mixed in wheat bran to initiate the reaction, and the reaction was carried out at ambient temperature in the dark for 72 h. Enzyme doses were 0 (control), 2.26, 4.52, 9.04, 18.04, and 221.6 U/g wheat bran (on dry weight basis) for each tested enzyme preparation with an initial treatment moisture content of 35% in all ground bran samples. The possible effects of moisture content on solid-state enzymatic reactions were examined using Viscozyme L at initial treatment moisture contents of 30 and 43% in the bran with enzyme doses of 0, 2.26, 4.52, 9.04, 18.09, and 33.91 U/g wheat bran (on dry weight basis). See Table 1 for enzyme characteristics. Enzymes were inactivated by heating using a microwave oven and re-ground to 40 mesh.

Sample Extraction Procedure. One gram samples of 40-mesh enzyme treated bran were extracted with 10 mL of 100% ethanol for 18 h under nitrogen in the dark at ambient temperatures. The ethanol extracts were used for ABTS$^{\bullet+}$ scavenging ability, oxygen radical absorbing capacity (ORAC), and DPPH$^{\bullet}$ scavenging activity assays. Known volumes of 100% ethanol extracts were dried under nitrogen, and the solid residue was quantitatively redissolved in DMSO for total phenolic contents (TPC) assay or acetone for the hydroxyl radical scavenging capacity assay (HOSC). Extracts were stored under nitrogen in the dark at ambient temperatures until further analysis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. ORAC assay was conducted with fluorescein (FL) as the fluorescent probe using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) according to a previous laboratory protocol (*16*) with modifications. Standards were prepared in 100% ethanol while all other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). The initial reaction mixture contained 225 μL of 8.16×10^{-8} M FL, 30 μL of antioxidant extract, standard, or 100% ethanol for blanks, and 25 μL of 0.36 M AAPH. FL and antioxidant extracts were mixed in a 96-well plate and preheated in plate reader for 20 min at 37 °C after which the AAPH

solution was added to initiate the antioxidant−radical reactions. The fluorescence of the assay mixture was recorded every minute for 80 min at 37 °C. Excitation and emission wavelengths were 485 and 535 nm, respectively. Results were expressed as μmoles of trolox equivalents (TE) per gram wheat bran on a dry weight basis.

Radical Cation ABTS$^{\bullet+}$ Scavenging Capacity. The free radical scavenging capacity of the 100% ethanol extracts was evaluated against ABTS$^{\bullet+}$ generated according to a previously reported protocol (*11*, *48*). Fifty microliters of the bran extracts were diluted to 500 μL with 100% ethanol to create working sample solutions. ABTS cation radicals were generated by oxidizing a 5 mM aqueous solution of ABTS with manganese dioxide for 30 min at ambient temperature. The final reaction mixture contained 80 μL of working sample solution or 100% ethanol for control, and 1.0 mL ABTS$^{\bullet+}$ solution with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured after a reaction time of 1 min. Trolox equivalents (TE) were calculated using a standard curve prepared with trolox and expressed in μmoles TE per gram of wheat bran on a dry weight basis.

Radical DPPH Scavenging Capacity. The DPPH$^{\bullet}$ scavenging capacity of the bran antioxidant extracts was determined following a previously reported procedure with modifications (*49*). Briefly, 100 μL of antioxidant extract or ethanol for blank was added to 100 μL of freshly prepared DPPH$^{\bullet}$ solution to initiate antioxidant−radical reaction. The absorbance of the reaction mixture was measured at 515 nm at 40 min of reaction. The initial concentration of DPPH$^{\bullet}$ was 100 μM for all reaction mixtures. DPPH$^{\bullet}$ radical scavenging capacity was expressed as the percent of DPPH$^{\bullet}$ scavenged in 40 min under the experimental conditions.

Hydroxyl Radical Scavenging Capacity (HOSC) Assay. HOSC assay was conducted with acetone solutions according to a previously published protocol (*23*) using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland). Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard or sample or blank, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 mM $FeCl_3$. Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Trolox prepared in acetone at concentrations of 20, 40, 60, 80, and 100 μM were used to prepare the standard curve for HOSC quantification. HOSC results were expressed as micromoles trolox equivalents (TE) per gram of wheat bran on a dry weight basis.

Total Phenolic Contents. The DMSO extracts were analyzed for total phenolic contents using the Folin−Ciocalteu reagent according to a previously reported procedure (*7*, *16*). Folin−Ciocalteu reagent was prepared by refluxing 85% phosphoric acid, sodium molybdate, sodium tungstate, and concentrated hydrochloric acid for 10 h, reacting with lithium sulfate, then oxidizing with bromine followed by filtration. The final reaction mixture contained 50 μL of antioxidant extracts in DMSO, 250 μL freshly prepared Folin−Ciocalteu reagent, 750 μL 20% sodium carbonate, and 3 mL of ultrapure water. Absorbance at 765 nm was read after a reaction time of 2 h at ambient temperature. Total phenolic contents were calculated using gallic acid as a standard.

Phenolic Acid Composition. Treated bran samples were analyzed for their soluble free, soluble conjugated, insoluble bound, and total phenolic acid compositions using a previously reported procedure (*16*). Acetone/methanol/water (7/7/6, v/v/v) was used to extract the soluble free and the soluble conjugated phenolic acids, while the insoluble bound phenolic acids remained in the resulting solid residue. The free and conjugated phenolic acids in the acetone/methanol/water solution were separated based on their solubility in ethyl acetate/ethyl ether (1:1, v/ v) under acidic condition (pH = 2). Soluble conjugated phenolic acids were also hydrolyzed using NaOH, and re-extracted in ethyl acetate/ethyl ether (1:1, v/v) after the reaction pH was brought to pH 2. The solid residue with insoluble bound phenolic acids was hydrolyzed with NaOH, and the supernatant was re-extracted with ethyl acetate/ethyl ether (1:1, v/v) after pH was adjusted to about pH 2. The concentration of NaOH in the hydrolysis reaction mixtures was 2 M. After evaporation of ethyl acetate and ethyl ether, each phenolic acid extract was quantitatively redissolved in MeOH and analyzed by HPLC using a Phenomenex C18 column (250 mm × 4.6 mm) according to an established protocol (*11*). Phenolic acids were separated using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v). Solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min (*11*, *16*). Identification of phenolic acids was accomplished by comparing the retention time of peaks in the samples to that of the standards under the same HPLC conditions. Quantification of each phenolic acid was determined using external standards and total area under each peak.

Moisture Content. The moisture content of bran samples before and after the solid-state enzymatic reactions were determined using an oven following the AACC method 44−16 (*50*).

Statistic Analysis. Data were reported as mean ± SD for triplicate determinations. ANOVA and Tukey's tests were performed (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) to identify differences among means. Statistical significance was declared at $P \leq 0.05$.

RESULTS AND DISUCUSSION

Wheat is an important dietary staple with over 600 million metric tons estimated to be produced worldwide in 2006 (*51*). One of the important antioxidants in wheat grain, phenolic acids, are concentrated in the bran fraction and are thought to contribute to the disease prevention properties attributed with wheat bran or whole grain consumption in epidemiological studies (*26*, *52*, *53*). The majority of the phenolic acids present in wheat, however, are only minimally available for absorption (bioaccessible) because they are bound to wheat bran cell wall materials in the aleurone and pericarp layers through ester linkages (*52*, *32*, *35*).

The polysaccharide composition of wheat bran aleurone and pericarp cell walls includes mostly arabinoxylans with some β-glucans and cellulose (*33*, *34*). Phenolic acids are primarily esterified to the C-5 hydroxyl group of α-L-arabinofuranosyl substituents which are linked to C-2 or C-3 on the xylopyranosyl backbone (*34*, *36*). Previous studies on the enzymatic breakdown of cell wall materials for release of phenolic acids have found the most effective enzyme mixtures to include xylanses, α-L-arabinofuranosidases, acetyl xylan esterases, α-glucoronidases, and ferulic and *p*-coumaric esterses (*39*). These studies have used either mixtures of purified enzymes and isolated enzymes from microorganisms, or they have used commercial mixed enzyme preparations such as Ultraflo-L, Viscozyme-L, Celluclast 1.5L, Termamyl, and Lallzyme in submerged aqueous reaction systems (*37*, *42*, *54*).

The present study utilizes a solid-state enzymatic reaction system to release the phenolic acids present in wheat bran. Solid-state reaction systems are used in the production of many food ingredients and have been researched for the enzymatic release of phenolics from materials such as soybean powders, pineapple residue, cranberry pomace, and black current juice residue (*45*, *47*, *55*, *56*). Solid-state enzymatic reaction systems are more commercially practical for food ingredient modification and production than the aqueous phase reaction systems because there is no additional step needed after inactivation of enzyme(s) to remove water or isolate the final products. In addition, the solid-state enzymatic procedure generates no waste and requires no special equipment.

Effects of Different Enzyme Preparations on the Antioxidant Properties of Hard Wheat Bran. Five commercial food-grade enzyme preparations (Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, and Celluclast 1.5L) and porcine liver esterase (a purified non-food-grade enzyme) were used to treat Akron and Jagalene hard wheat bran at doses of 0−18 units per gram under the solid-state reaction conditions. All enzymes were additionally tested at a dose of 221 U/g,

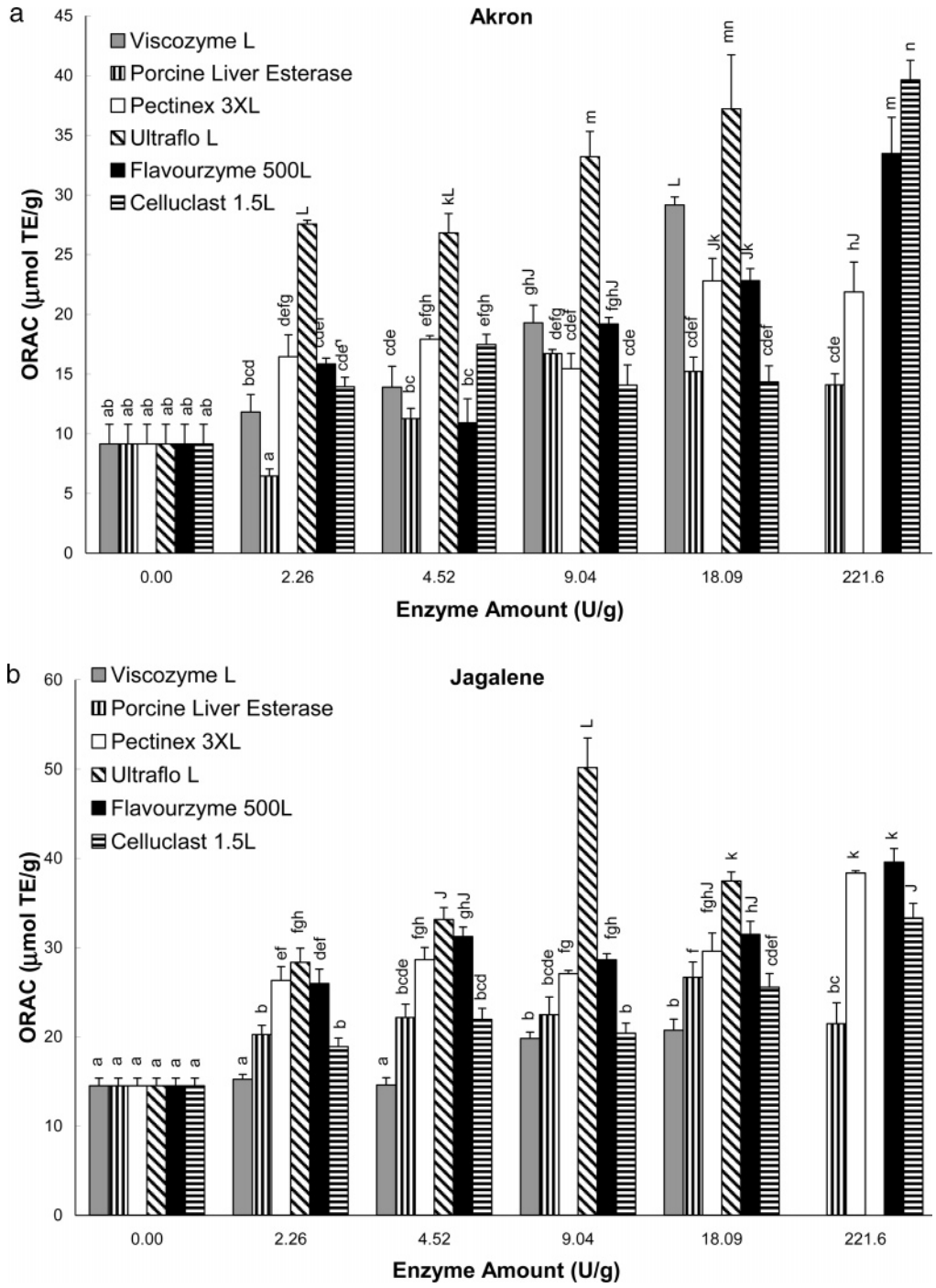

Figure 1. Effects of different enzyme treatments on the oxygen radical absorbing capacities (ORAC) for two hard wheat bran varieties. (**a**) Akron wheat bran; (**b**) Jagalene wheat bran. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as μmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

except Viscozyme L and Ultraflo L preparations which were not concentrated enough to allow this high dosage. To increase the scope of results for this study, bran samples of two hard wheat varieties were included. Statistical analysis for each antioxidant assay was performed between treatments within each variety to determine any significant differences. Individual enzyme preparations differed in their effects on bran antioxidant properties including oxygen radical absorbing capacity (ORAC), $ABTS^{\bullet+}$ scavenging capacity, $DPPH^{\bullet}$ scavenging capacity, and total phenolic contents.

Oxygen Radical Absorbing Capacities (ORAC). ORAC measures peroxyl radical scavenging capacity expressed as μmoles trolox per gram wheat bran on a dry weight basis. Solid-state enzymatic treatment significantly increased ORAC values of both Akron and Jagalene wheat bran samples (**Figure 1a**,**b**). The largest increases in ORAC value versus the control were 4.3-fold for Akron wheat bran treated with 221 U/g Celluclast 1.5L and a 3.5-fold increase for Jagalene treated with 9 U/g Ultraflo-L. The most efficient enzyme, showing the greatest percent increase per unit of enzyme activity, was Ultraflo L for

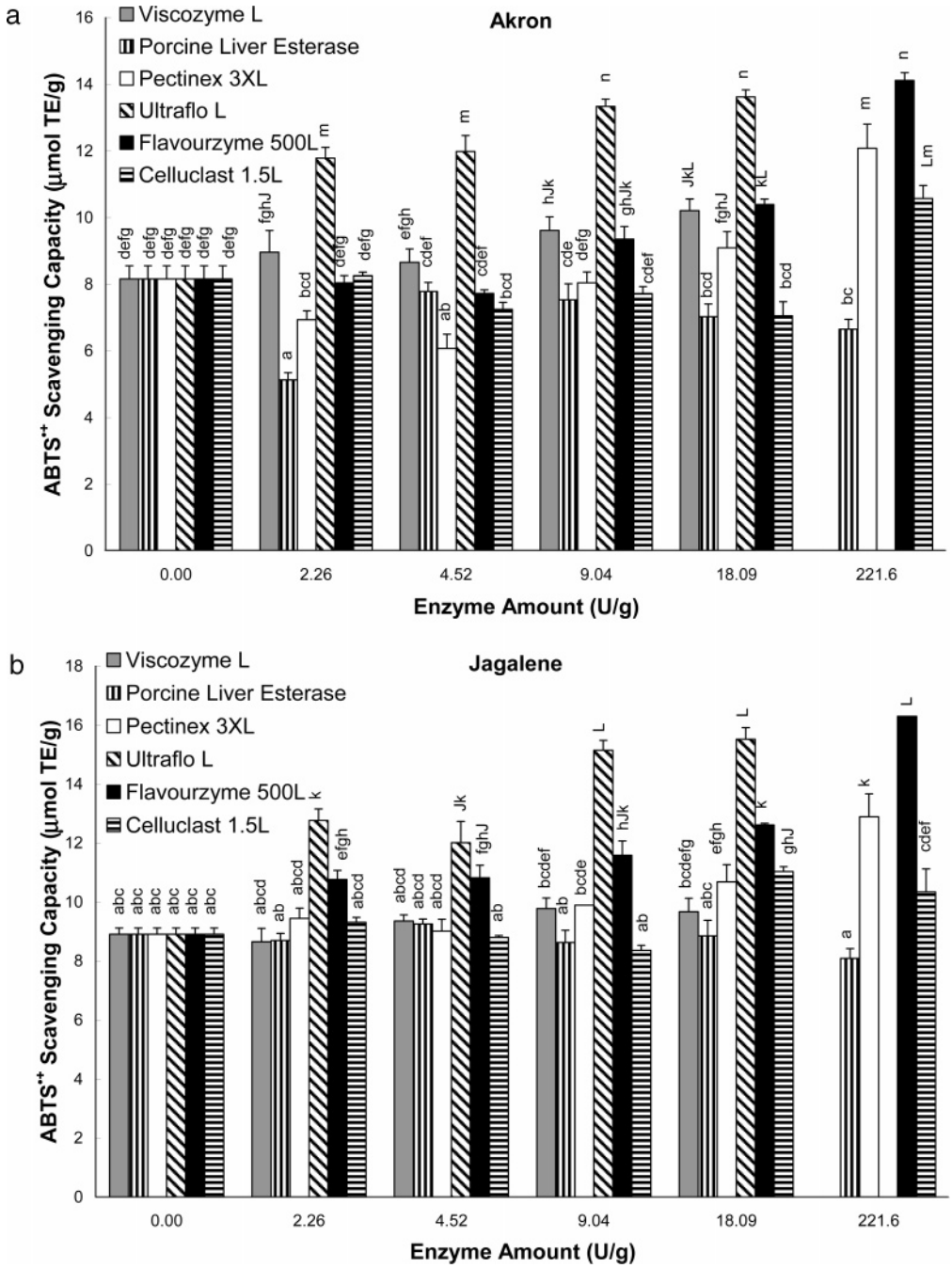

Figure 2. Effects of different enzyme treatments on the ABTS$^{\bullet+}$ scavenging capacities for two hard wheat bran varieties. (**a**) Akron wheat bran; (**b**) Jagalene wheat bran. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as μmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

bran samples of both wheat varieties. Other enzyme preparations showed ORAC value increases of 1.8-, 2.4-, 4.0-, and 3.6-fold for Akron wheat bran treated with porcine esterase, Pectinex 3XL, Ultraflo L, and Flavourzyme 500L, respectively, while treatments of Jagalene bran showed increases of 1.4-, 1.8-, 2.6-, 2.7-, and 2.3-fold for Viscozyme L, porcine esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L, respectively. Results for all treatments indicated that ORAC value increases for enzyme treatments were dose dependent, except for Pectinex 3XL treatments.

ABTS$^{\bullet+}$ Scavenging Capacities. ABTS$^{\bullet+}$ scavenging capacities of enzyme-treated bran samples were measured and expressed as μmoles trolox per gram bran on a dry weight basis. Some enzyme treatments resulted in significant increases in ABTS$^{\bullet+}$ scavenging capacity of the bran (**Figure 2a**,**b**). For both Akron and Jagalene wheat bran samples, Ultraflo L was the most efficient enzyme preparation showing the greatest increases of ABTS$^{\bullet+}$ scavenging capacity on a per unit of enzyme activity basis, and it also showed the highest increases (1.7-fold) at 18 U/g dosage level. Flavourzyme 500L treatments showed comparable increases, but at the high enzyme level of 221 U/g. For both bran samples, porcine esterase treatments showed some significant decreases in ABTS$^{\bullet+}$ scavenging capacity versus control at low concentrations, and no significant

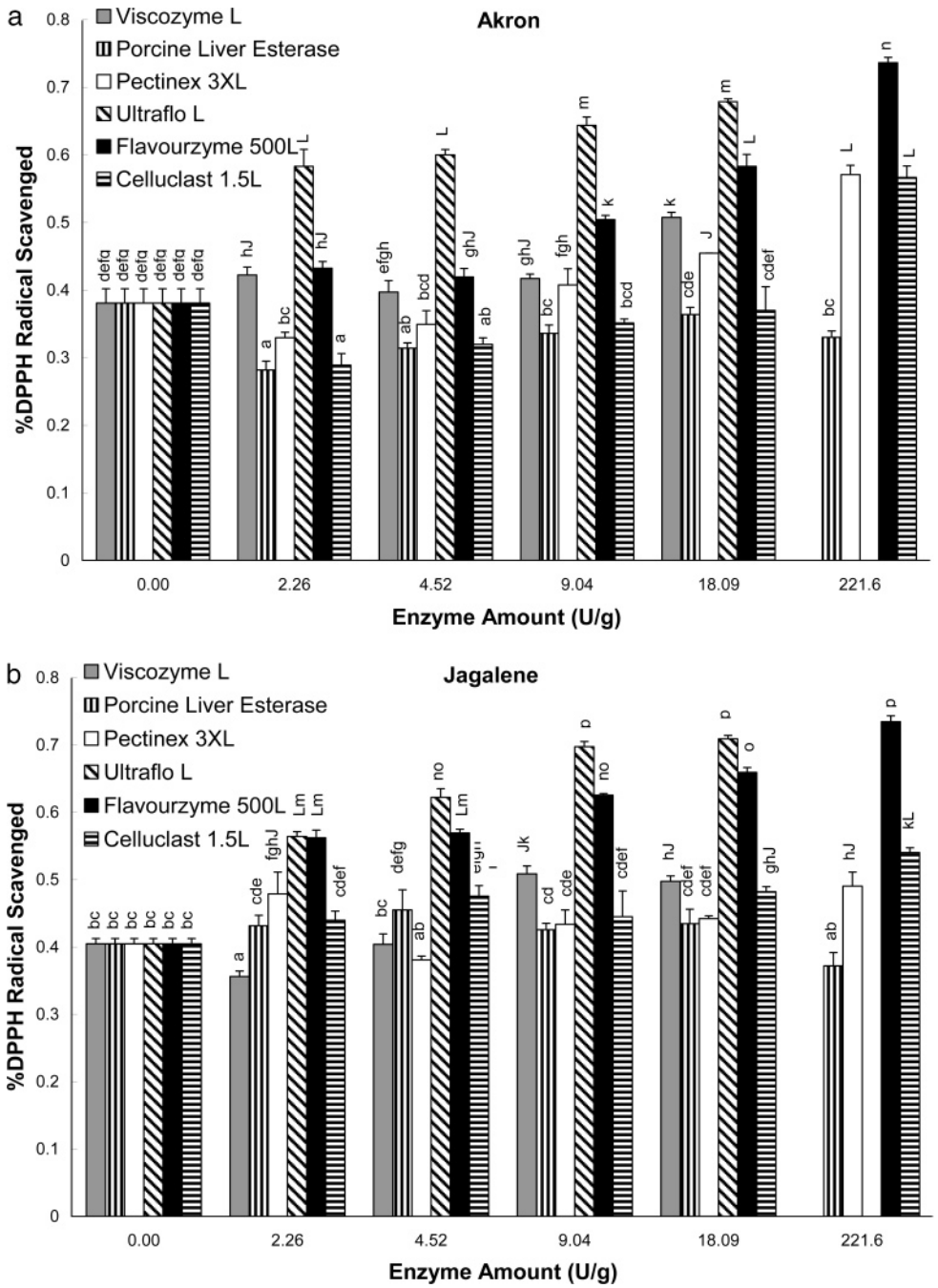

Figure 3. Effects of different enzyme treatments on the DPPH• scavenging capacities for two hard wheat bran varieties. (**a**) Akron wheat bran; (**b**) Jagalene wheat bran. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as percent DPPH• scavenged after 40 min reaction time. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

differences from control at other levels. Other enzymes for both varieties showed some small significant increases, but only at higher enzyme levels. Results in **Figure 2** also indicated that $ABTS^{\bullet+}$ scavenging capacity increases for enzyme treatments were dose dependent except for Celluclast 1.5L treatments.

DPPH• Scavenging Capacities. DPPH• scavenging capacities of the enzyme-treated wheat bran samples are shown in **Figure 3a**,**b** expressed as percent DPPH• scavenged. For treatments of Akron bran, Ultraflo L was the most efficient enzyme preparation and had the highest increases of 1.8-fold at 18 U/g dosage, while Flavourzyme 500L treatments showed comparable increases, but at higher enzyme doses. For Jagalene treatments, both Ultraflo L and Flavourzyme 500L showed similar efficiencies with the greatest percent increases of 1.8-fold at 18 and 221 U/g, respectively. Porcine esterase treatments for both varieties showed no significant increases versus the control, and for Akron at 2 U/g a significant decrease in DPPH• scavenging capacity was shown. Other enzyme treatments for both varieties showed some small increases in DPPH• scavenging capacities at higher enzyme levels. Results for all treatments indicated that DPPH• scavenging capacity increases for enzyme treatments were dose dependent except for porcine esterase treatments for Jagalene bran. In addition, Cellulcast 1.5L treatment decreased DPPH• scavenging capacity in Akron bran, but dose-dependently

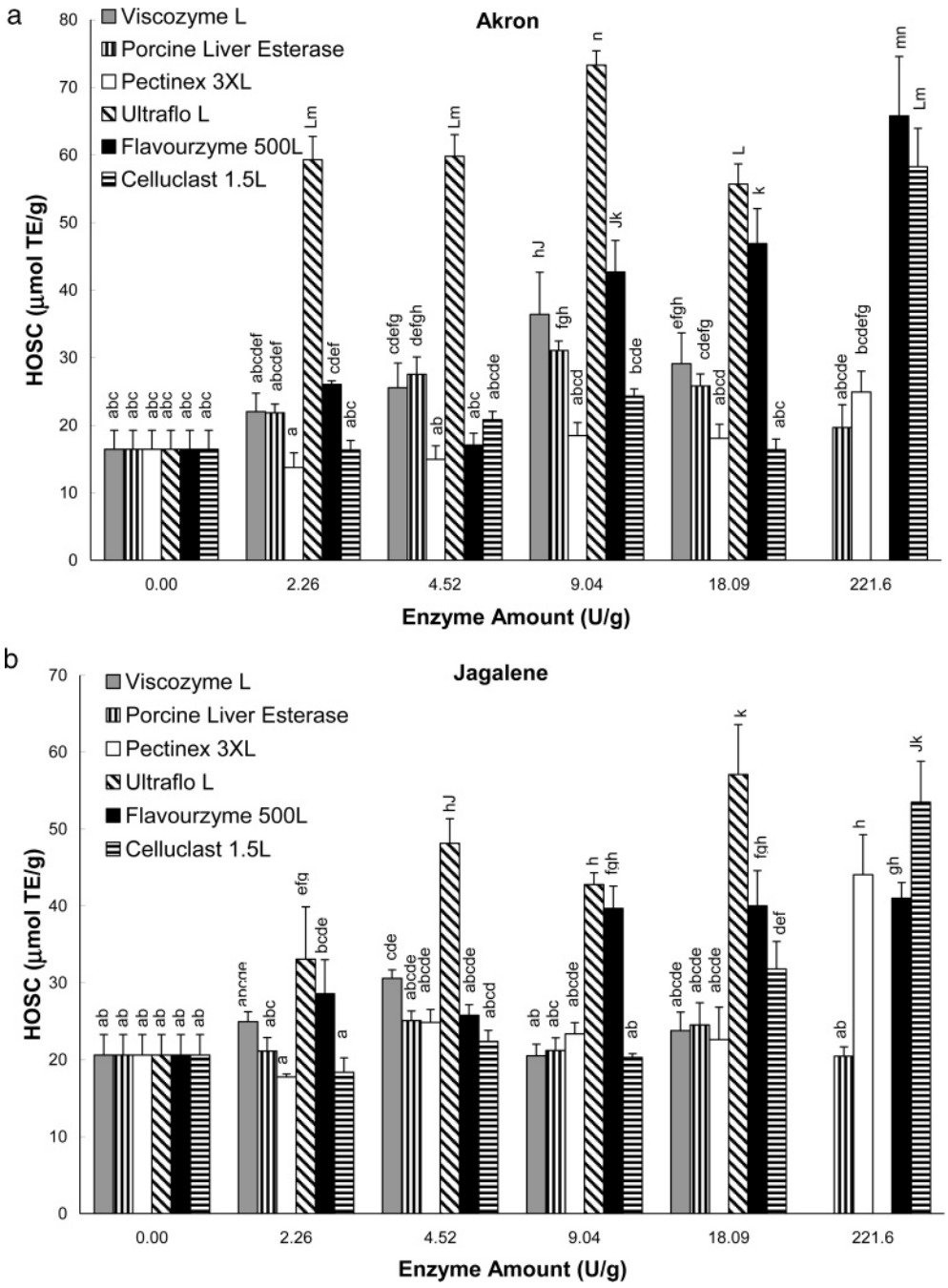

Figure 4. Effects of different enzyme treatments on the hydroxyl radical scavenging capacities (HOSC) for two hard wheat bran varieties. (**a**) Akron wheat bran; (**b**) Jagalene wheat bran. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activity for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as μmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

increased DPPH$^{\bullet}$ scavenging capacity in Jagalene bran (**Figure 3**), suggesting that the bran sample may respond to a selected enzyme differently.

Hydroxyl Radical Scavenging Capacity (HOSC). The hydroxyl radical scavenging capacities of enzyme-treated wheat bran samples are shown in **Figure 4a**,**b** with results expressed as μmoles trolox equivalents per gram wheat bran on a dry weight basis. Results showed some significant increases in HOSC values for both varieties of wheat bran, dependent on enzyme preparation and dose. The largest increases in HOSC versus control were Ultraflo L treatments for both varieties of wheat bran, showing 4.5- and 2.76-fold for Arkon at 9 U/g and Jagalene at 18 U/g. Ultraflo L was also observed to be the most efficient enzyme preparation tested, with the greatest increase in hydroxyl radical scavenging capacity per unit of enzyme. Other enzyme preparations showed maximum increases in HOSC for Akron of 2.2-, 1.9-, 1.5-, 4.0-, and 3.5-fold for Viscozyme L, Porcine Esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L. Treatments of Jagalene bran showed increases of 1.5-, 1.2-, 2.1-, 2.0-, and 2.5-fold for Viscozyme L, porcine esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L, respectively.

Total Phenolic Contents. Total phenolic contents (TPC) were measured for enzyme treated bran sample using the Folin−

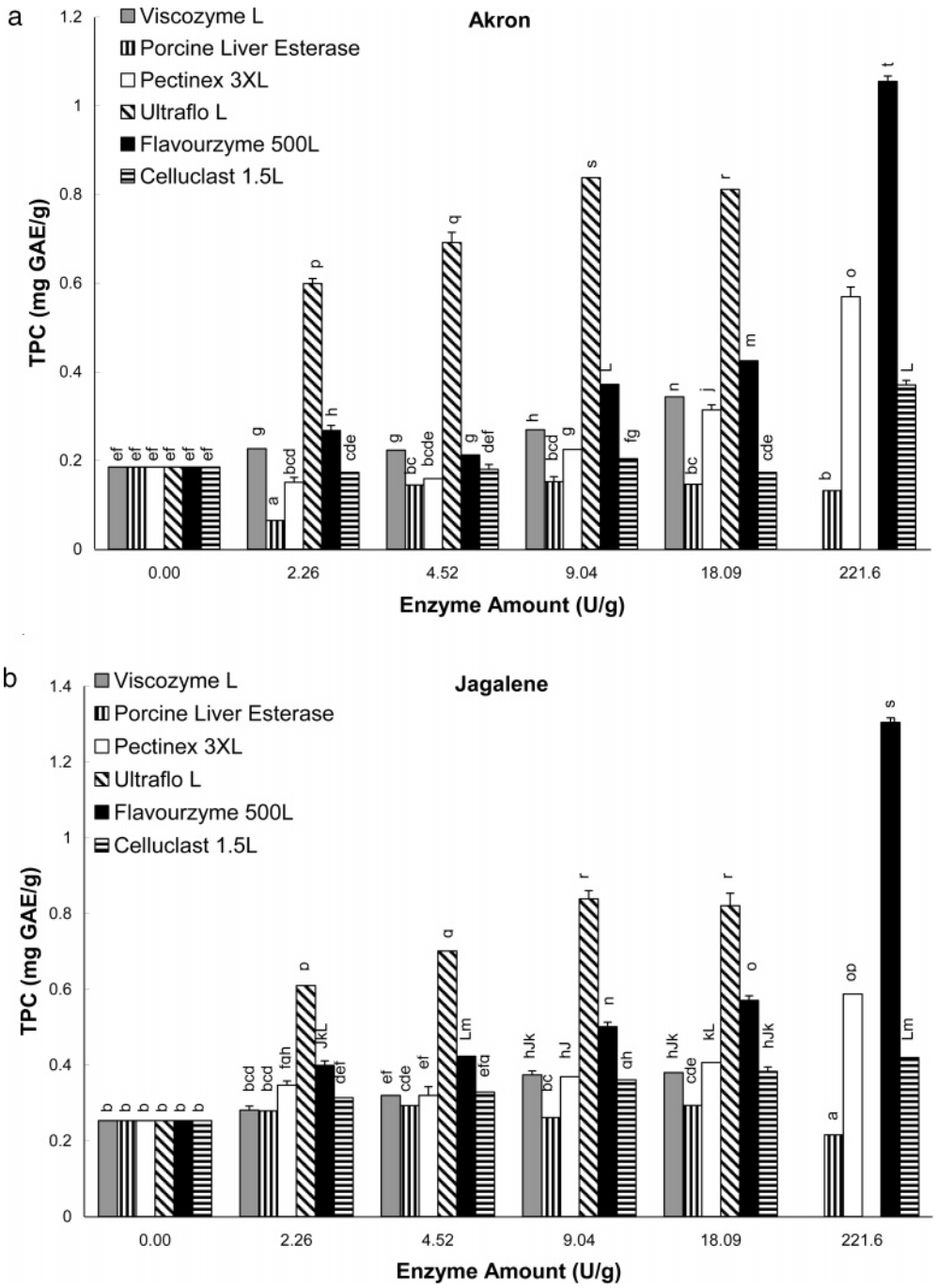

Figure 5. Effects of different enzyme treatments on the total phenolic content (TPC) for two hard wheat bran varieties. (**a**) Akron wheat bran; (**b**) Jagalene wheat bran. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as milligram gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Ciocalteu reagent, and expressed as milligrams of gallic acid equivalents per gram of bran on a dry weight basis. Individual enzyme preparation differed in their capacity to enhance the releasable levels of total phenolic compounds from Akron and Jagalene bran samples (**Figure 5a**,**b**). For both Akron and Jagalene wheat bran samples Ultraflo L was the most efficient enzyme preparation, showing the greatest capacity to increase releasable TPC per unit of enzyme activity, and showed maximum increases of 4.5- and 3.3-fold at the enzyme concentration of 9 U/g, respectively. Regardless of wheat variety, increasing enzyme concentration in the solid-enzymatic reaction mixture from 0 to 9 U/g increased the extractable amount of TPC, but further increase of Ultraflo L concentration from 9 to 18 U/g did not increase the level of releasable TPC under the experimental conditions. Flavourzyme 500L treatments also dose-dependently increased the amount of extractable TPC in both Akron and Jagalene wheat bran samples under the experimental conditions (**Figure 5**). Flavourzyme 500L treatments at a level of 221 U/g resulted in the greatest increases of TPC, with 5.6- and 5.1-fold increases for Akron and Jagalene, respectively. Porcine liver esterase treatments were not able to improve the TPC release from either Akron or Jagalene wheat

bran samples, under testing conditions, and led to decreased TPC for Akron bran at 2 and 4 U/g (**Figure 5**). Viscozyme L, Pectinex 3XL, and Celluclast 1.5L were able to slightly and dose-dependently enhance the extractable TPC levels (**Figure 5**).

The results of these antioxidant activity evaluations show for the first time that solid-state enzyme treatments may increase the in vitro antioxidant activities of wheat bran. These increases in antioxidant activity are likely a result of the enzymatic release of the insoluble bound phenolic acids, a major form of antioxidant compounds in wheat. These results also suggest that Ultraflo-L may be more efficient, on per enzyme activity unit basis, than other tested enzyme preparations for enhancing the extractable antioxidant components and thereby increasing in vitro antioxidant activities of wheat bran. Ultraflo L is considered as a well-balanced mixture of cell wall degrading enzymes including β-glucanase as the primary enzyme activity and other reported side activities including arabanase, cellulase, pentosanase, xylanase to degrade the xylan backbone, and importantly significant feruloyl esterase activity to release bound ferulic acid, the predominant phenolic acid in wheat (*42*). Interestingly, Flavourzyme 500L, a peptidase, was able to significantly increase the releasability of bran antioxidants under the solid-state enzymatic reaction conditions, suggesting that this enzyme preparation may be capable of hydrolyzing ester and/or glycosidic bonds. The purified esterase (porcine liver esterase) could not significantly increase extractable antioxidant activities at all tested concentrations or antioxidant properties, suggesting that this esterase may not be able to specifically hydrolyze hydroxycinnamate ester bonds in wheat bran due to the possible steric hindrance of the polysaccharide structure and the limited migration of enzyme under the solid-state reaction conditions. Solid-state reaction with Pectinex 3XL, a preparation with primary polygalacturonase activity, might significantly increase the releasable antioxidant properties, but mostly at high concentrations indicating that the reported side activities of the enzyme preparation such as the xylanase and hemicellulase activities (see **Table 1**) may be responsible for these increases. Viscozyme L and Celluclast 1.5L are two commonly used cell wall degrading enzyme preparations, Celluclast containing primarily cellulase activity while Viscozyme L has mixed xylanase, hemicellulase, and cellulase activities. Viscozyme L treatment resulted in significant increases in antioxidant activity, but not as effective as Ultraflo L on per enzyme unit basis under the solid-state reaction conditions. This is supported by a study from Bartolomé and others (*54*) showing Ultraflo L to be superior to Viscozyme L and other enzyme preparations for enhancing the release of phenolic acids from barley using aqueous phase reactions. Celluclast 1.5L treatment also resulted in some significant increases in antioxidant activities, but mostly at higher enzyme doses. This result agrees with the fact that the wheat bran cell wall matrix contains mostly arabinoxylans which cannot be hydrolyzed by cellulases, but at high concentrations of Celluclast 1.5L, its side activities may be able to hydrolyze the matrix.

The above results comparing different enzyme preparations suggest that primary enzyme activity in addition to side activities (accessory enzymes) play an important role in determining the ability of an enzyme preparation to increase the extractable antioxidant properties of wheat bran. This is supported by numerous studies which have suggested that wheat bran main-chain (xylan) depolymerizing enzymes work in combination or possible synergy with side-group cleaving accessory enzymes such as arabinases and ferulic acid esterases to provide the most effective enzyme system for breaking down cell wall material and releasing ferulic acid from wheat bran (*39*, *42*). This can be explained in that hydrolysis of the xylan backbone of wheat bran cell wall material enhances the accessibility of ferulic acid esterases and arabinases to their substrates and vice versa, providing a synergistic effect. Other studies have also suggested that the different types of xylanases, which have different specificities for xylan hydrolysis dependent on substitution of the backbone, can significantly alter the effectiveness of these enzyme systems for degrading wheat bran cell wall materials (*42*). Last, a study by Sørensen and others (*37*) showed that xylanases present in Ultraflo L and Celluclast 1.5L enzyme preparations may have synergistic interactions in their abilities to hydrolyze wheat bran arabinoxylans, suggesting a possible synergism between these two preparations in releasing phenolic acids release from wheat bran. Together, the results of this and other studies suggest the opportunity for evaluating the synergistic effects of different enzyme combinations in releasing extractable antioxidants from wheat bran in solid-state reaction systems.

Soluble and Insoluble Bound Phenolic Acid Compositions. The effects of solid-state enzymatic treatments on total soluble and insoluble bound phenolic acid compositions in wheat bran were investigated using the Viscozyme L and Ultraflo L enzyme preparations. The total soluble including soluble free and conjugated, and insoluble bound phenolic acid compositions of the selected treatment samples were determined using HPLC and compared to that of a control sample which went through the solid-state reaction procedure without enzyme addition. Results shown in **Figure 6a** show the changes in individual phenolic acids for Viscozyme L treatments of Akron wheat bran at 0 (control), 4.5, and 18 U/g enzyme levels. **Figure 6b** shows Ultraflo L treatments of Akron wheat bran at 0 (control), 2, and 18 U/g. Significant concentrations of four phenolic acids were detected in treated and control samples including vanillic, syringic, *P*-coumaric, and ferulic acids. All analyzed treatments showed no detectable soluble conjugated phenolic acids. For Viscozyme L treatments, bound phenolic acids decreased dependent on enzyme level with individual ranges of 22−14, 21−13, 38−21, and 1200 to 635 μg/g bran for vanillic, syringic, *p*-coumaric, and ferulic acids respectively, while free phenolic acids increased dose-dependently with ranges of 21−40, 3.2−11, and 7−95 μg/g bran for vanillic, syringic, and ferulic acids. Similar dose dependencies and concentration ranges for bound and free phenolic acids in Ultraflo L treatments were observed except for free ferulic acid which had significantly higher concentrations of 433 and 173 μg/g bran at 2 and 18 U/g treatment levels indicating decreased free ferulic concentrations with increased enzyme levels.

Results for these two commercial enzyme preparations to release ferulic and vanillic acids from wheat bran samples indicate that Ultraflo L hydrolyzed more insoluble bound phenolic acids than Viscozyme L. These results agree with the antioxidant property results in **Figures 1−4** which indicate that Ultraflo L was superior to Viscozyme L in improving the extractable antioxidant properties of both wheat bran samples. The HPLC results were also supported by a previous study (*54*) which found that Ultraflo L was able to release more ferulic and *p*-coumaric acids from barley spent grain than Viscozyme L. In addition, these results were supported by a study conducted by Sørensen and other (*37*) which showed that Ultraflo L was superior to Viscozyme L at hydrolyzing wheat arabinoxylans which are thought to the main polysaccharide matrix to which phenolic acids are bound to in wheat bran.

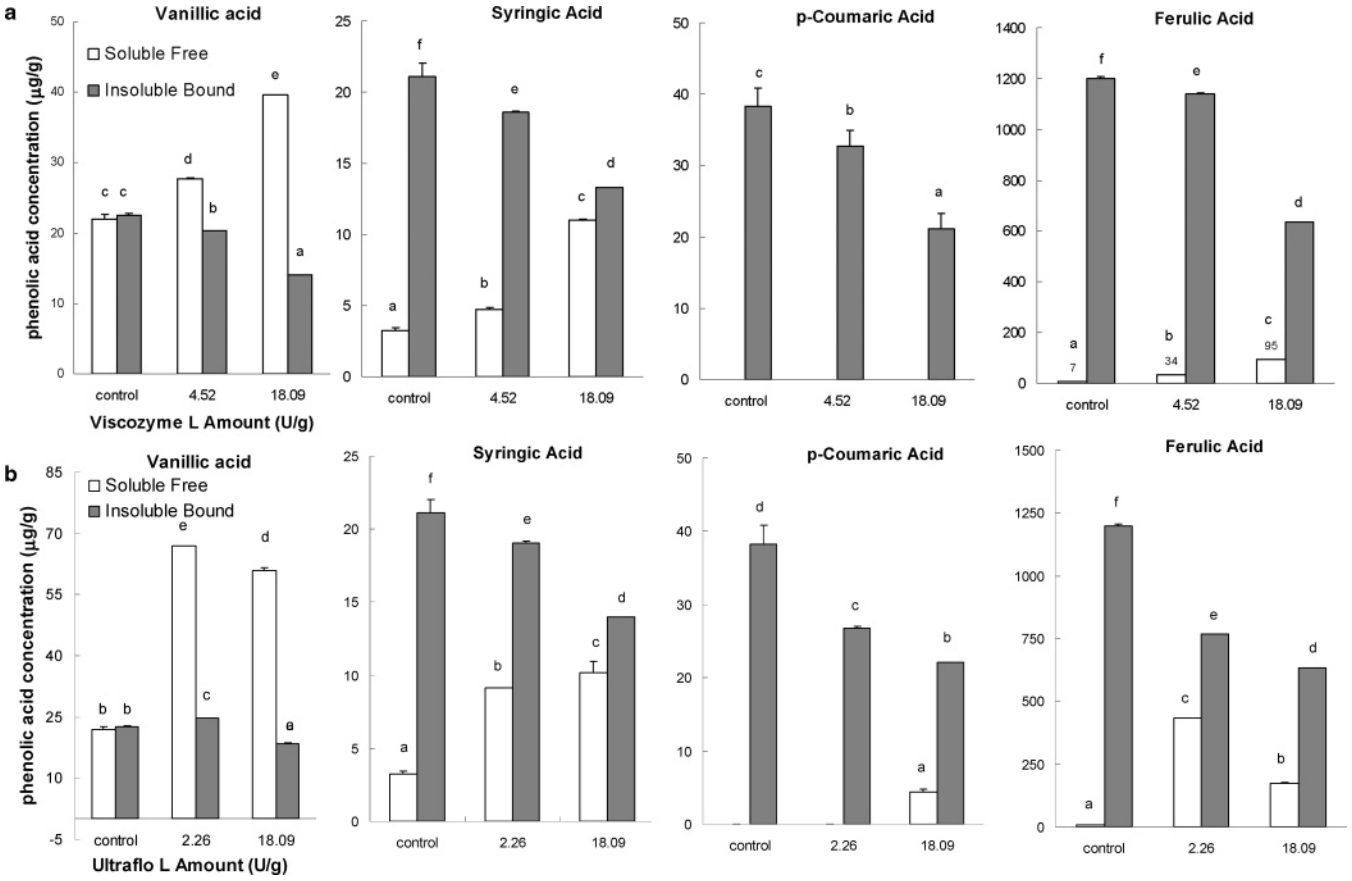

Figure 6. Effects of Viscozyme L and Ultraflo L treatments on the phenolic acid composition of Akron wheat bran. (**a**) Viscozyme L treatments; (**b**)Ultraflo L treatments. Enzyme treatments of bran were carried out at a moisture content of 35% for all treatments. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See **Table 1** for enzyme characteristics. Results are expressed as micrograms of individual phenolic acids per gram of wheat bran on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

The percent bound ferulic acid release from wheat bran found in this study using the most effective enzyme preparation, Ultraflo L, ranged from 22 to 50% at the enzyme concentrations evaluated in this study (**Figure 6b**). These results, while lower than those from a previous study using an aqueous phase enzyme reaction, which showed 90% release using the same enzyme preparation on wheat bran (*42*), demonstrate significant potential for application of these solid-state reactions commercially where aqueous phase reactions are not viable.

In particular, this release of free phenolic acids may have applications in improving the bioaccessibility and therefore bioavailability of wheat bran phenolics. It is well accepted that soluble free phenolic acids are readily bioaccessible in the human small intestine and are a major contributor to the absorbable phenolics present in wheat (*57*). The majority of wheat phenolics, however, are present in insoluble bound forms which are thought to be bioaccessible only in colon where microflora could hydrolyze them to free phenolics followed by further metabolism or absorption in the colon (*16*, *57*). A recent human study by Kern and others (*35*) which evaluated the recovery of phenolic acids in plasma and urine after wheat bran consumption found only 3% of the total phenolics (soluble free, soluble conjugated, and insoluble bound) were absorbed after 24 h. This study also concluded that the site of absorption was primarily the small intestine, with maximum absorption 1 to 3 h after ingestion. This and other numerous studies have therefore concluded that the low bioavailability of wheat phenolics is due to its primarily insoluble bound form in wheat bran (*35*, *57*, *58*). The results of this study showed significant conversion of insoluble bound phenolics to soluble free phenolics, thereby demonstrating the potential to improve the bioaccessibility and potential bioavailability of wheat phenolic acids.

Influence of Moisture Content (MC) on the Solid-State Enzymatic Reaction. Water is a required agent in hydrolysis reactions including those catalyzed by esterases and glycosidases. The amount of free water may be limited under the solid-state enzymatic reaction conditions, and thus it may alter the overall effectiveness of enzyme treatments because of the reduced mobility of enzyme and reactant molecules and the availability of water molecules as a reactant. To evaluate the potential effect of MC on the efficacy of enzyme treatments, Akron wheat bran was treated with Viscozyme L, a widely available and popular cell wall degrading enzyme preparation used by food manufacturers, at 30% and 43% MC levels under the solid-state reaction conditions using five concentrations of enzyme. Multiple enzyme concentrations were used to obtain a general conclusion. The enzyme concentrations were from 2 to 34 units per gram dry wheat bran. The enzyme treated bran samples were extracted with ethanol and evaluated for their antioxidant properties measured as ORAC, DPPH$^{\bullet}$ scavenging capacity, and total phenolic content. MC had a significant effect on wheat bran antioxidant property changes as a result of solid-state enzyme treatments (**Figures 7**−**9**).

Results in **Figure 7** indicate that Viscozyme L treatments at

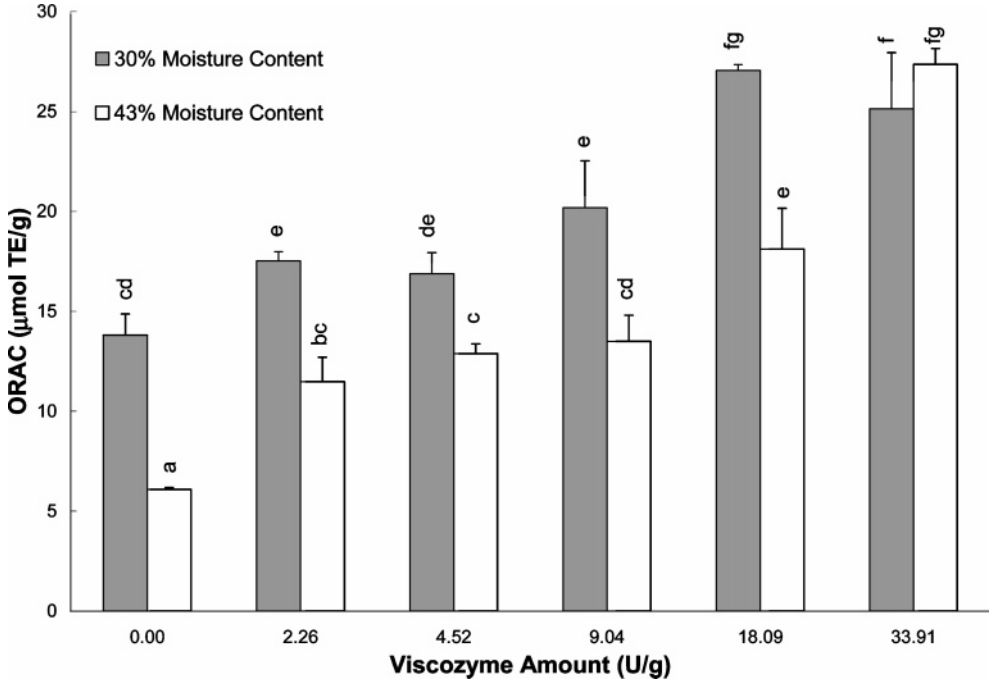

Figure 7. Effects of moisture content on the oxygen radical absorbing capacities (ORAC) of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See **Table 1** for enzyme characteristics. Results are expressed as μmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

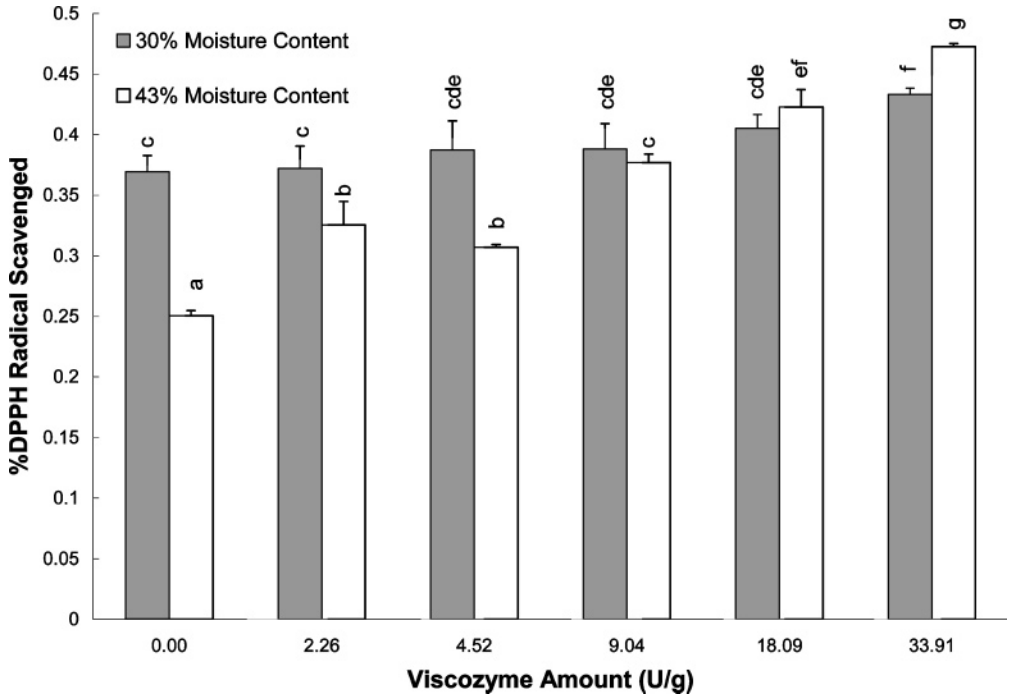

Figure 8. Effects of moisture content on the DPPH• scavenging capacities of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See **Table 1** for enzyme characteristics. Results are expressed as percent DPPH• scavenged after 40 min reaction time. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

30% MC resulted in significantly higher ORAC values than that at 43% MC for all tested enzyme concentrations except the highest enzyme concentration (34 U/g). The bran samples treated with 18 U/g Viscozyme L at two MCs had the largest difference in their ORAC values, which was about 1.5-fold. **Figure 8** indicates that at low enzyme concentration (2.0 − 4.5 U/g) bran samples treated at 30% MC had significantly higher DPPH• scavenging capacity values than those treated with the same level of enzyme at 43% MC, while MC had no significant effects when Viscozyme L concentration was 9 and 18 U/g in the solid-reaction mixtures. At the 33 U/g level, however, the bran sample treated with Viscozyme L at 43%

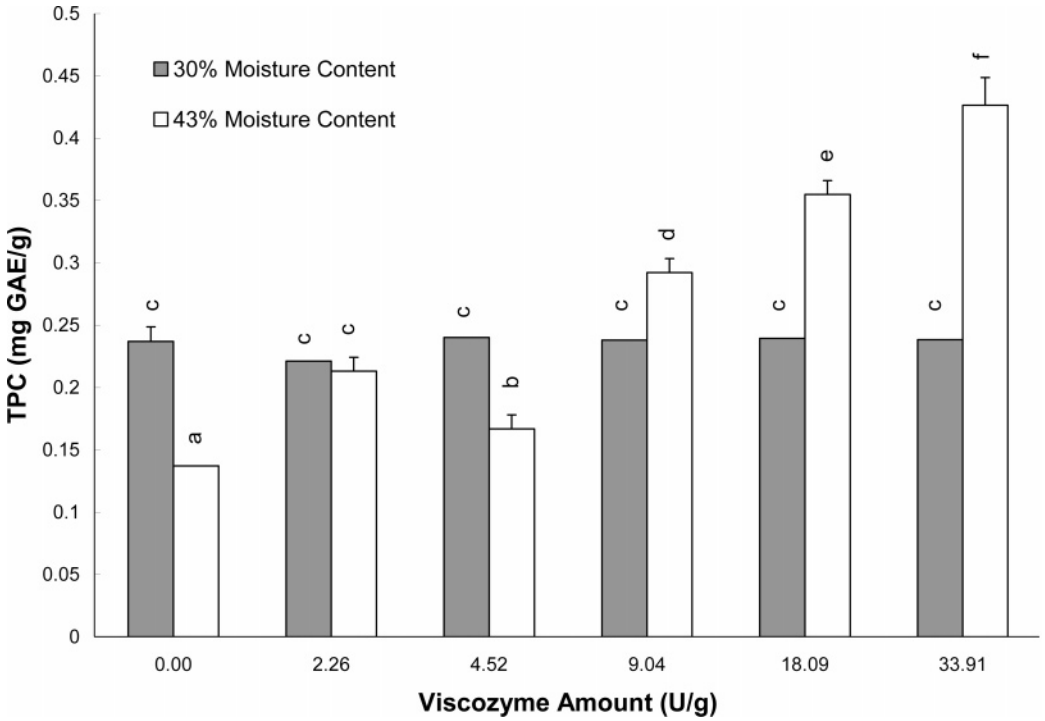

Figure 9. Effects of moisture content on the total phenolic contents of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages are expressed as enzyme activity units per gram of wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See **Table 1** for enzyme characteristics. Results are expressed as milligram gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

MC had a significantly larger DPPH$^{\bullet}$ scavenging capacity (**Figure 8**). The TPC values of Akron bran treated with the same concentrations of Viscozyme L at two moisture contents were determined and compared in **Figure 9** on a per dry bran weight basis. Similar to that observed in **Figure 7** and **8**, the effects of moisture content on solid-state enzymatic treatments depended on the enzyme concentration in the reaction mixture. When the moisture content was 30%, bran samples treated with an enzyme level of 0−4.5 U/g had significantly higher or the same level of extractable TPC, whereas the bran samples treated with enzyme levels of 9−34 U/g at 43% MC had higher TPC values than those reacted at 30% MC.

Taken together the trend of these results suggest that for solid-state Viscozyme L treatments of wheat bran, the enzyme reactions are affected by moisture content in the reaction system, and the effects are dependent on enzyme concentrations. At lower enzyme levels, the solid-state enzymatic treatments at lower MC resulted in higher extractable antioxidant activities, while the opposite was true at high enzyme doses. These results may be explained on the basis that at low enzyme concentrations, less water is required as a reactant to reach the maximum velocity of the reaction and additional free water may dilute the local enzyme concentration and further reduce the maximum enzyme reaction velocity. This reduction of maximum enzyme reaction velocity could then lead to the decrease in total extractable antioxidant activities or TPC values shown in this study when MC was increased from 30% to 43% under the solid-state enzymatic reaction conditions. On the other hand, when enzyme concentration was high in the reaction mixture, more free water was required to participate in the hydrolysis reaction. In other words, Viscozyme L was not saturated with the local free water molecules when enzyme concentration was high, and an increase in moisture content from 30% to 43% significantly enhanced the enzyme reaction and resulted in more extractable antioxidants. It needs to be pointed out that increasing the level of enzyme or moisture content in the solid-state enzymatic reaction system may significantly elevate the overall cost of the processing. Additional research is suggested to further understand the interaction of moisture and enzyme dose effects on improving wheat bran antioxidant properties. Further research is required to optimize the procedure before it may be utilized for commercial applications.

Conclusion. Our results showed for the first time that solid-state enzyme reactions can be utilized to enhance the extractable antioxidant properties and the potential bioavailability of antioxidants in wheat bran, which may improve its health benefits as well as its commercial value and market competitiveness. The increases in antioxidant properties were shown to be dependent on both the type of enzyme preparation and its dose. This research also showed that the effects of reaction moisture content during solid-state treatments are dependent on the enzyme dosage level. The solid-state enzymatic procedure used in this study requires no special equipment and involves no chemicals, and it may have potential for commercial applications. Lastly, this study points to future research opportunities in evaluating and developing more effective enzyme preparations for improving the bioaccessibility of antioxidants in wheat bran and other cereal grain based food and nutraceutical ingredients.

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