

Effects of Arabinoxylans on Activation of Murine Macrophages and Growth Performance of Broiler Chicks

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ABSTRACT

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Arabinoxylans occur in a wide variety of agricultural products and may contribute a significant portion of human dietary fiber intake. Corn hulls and banana peels are potential sources of arabinoxylans with isolation yields of ≈ 40 and 10% when extracted with dilute alkali. A broiler chick growth study was performed to determine the effect of extracted corn hull arabinoxylan on performance and attachment of *Salmonella*, as a representative of an enteric pathogen, to the ileum. Ability of arabinoxylans to

activate a macrophage cell line as an immune stimulator was determined by respiratory burst assay. Corn hull arabinoxylan tended to increase body weight gain and reduced attachment of *Salmonella* to ileal tissue in broiler chicks undergoing mild heat stress. Arabinoxylans from corn hulls and banana peels showed positive oxidative burst in macrophage cells. Collectively, these data indicate the two arabinoxylans have the potential to be used as health-promoting dietary supplements.

Beneficial health effects of hemicelluloses include improving lipid metabolism and mineral balance (Lopez et al 1999), improving colon function (Lu et al 2000), protection against colon cancer (Poutanen et al 1998), reducing the risk of heart disease and generally improving body health (McPherson 1993). Hemicelluloses from various sources have potential health benefits as an immunity enhancer (Kelly 1999; Nagata et al 2001).

Arabinoxylans, a class of hemicellulose, occur in a wide variety of cereal crops. Arabinoxylans may contribute a significant portion of human dietary fiber intake (Holloway et al 1980). Corn hulls are available as by-products from corn milling where over 4 million tons are produced per year in the United States (Hicks et al 2003). Currently, corn hulls have little recognized economic value and sometimes constitute a waste disposal problem (Doner and Hicks 1997) but they represent a low-cost resource that might present an industrial opportunity through further processing into value-added product. Corn hulls are a potential source of arabinoxylans with isolation yields of 30–40% through a cost-effective extraction with dilute alkali (Zhang et al 2003). Another potential source of arabinoxylans are bananas. About one fifth of bananas harvested are culled because they are slightly damaged or are too small for sale and disposal of this biomass is an environmental problem. This large quantity of cull bananas represents a potential resource for starch and hemicelluloses. Banana starch has great potential as a commodity due to its quality and availability from culls (Whistler 1998). After starch production, there remains potential for isolation and utilization of the hemicellulose in the residue peels. Hence, the arabinoxylan hemicelluloses from corn hulls and banana peels have been isolated, and their ability to activate a macrophage cell line as an indicator of immune stimulation has been determined. A broiler chick growth study was performed to determine the effect of corn hull arabinoxylan on performance and attachment of *Salmonella*, as a representative of an enteric pathogen, to the ileum.

MATERIALS AND METHODS

Hemicelluloses

Isolation and characterization of corn hull arabinoxylan has been reported previously (Zhang et al 2003). Banana peels obtained from the residue of starch production (Whistler 1998) were ex-

tracted in oxygen-free 4% sodium hydroxide solution at 25°C for 18–24 hr. Insolubles were removed by centrifugation, and alkaline supernatant was then combined and adjusted to pH 4–5 to precipitate hemicellulose A. The neutralized supernatant was then precipitated with 95% ethanol to give hemicellulose B. Both hemicelluloses were washed with ethanol and dried. Commercial defatted olives were extracted with the procedure discussed above to yield olive hemicelluloses. Arabinogalactan was extracted from larch wood (ClearTrac AG-99, Larex, St. Paul, MN).

Characterization

Hemicelluloses were hydrolyzed in 0.5M sulfuric acid at 100°C for 8 hr. Hydrolyzates were neutralized with barium carbonate, filtered, centrifuged, and dried. Monosaccharides were separated using a pulsed amperometric detector (Dionex BioLc HPLC) (Zhang et al 2003). Size-exclusion chromatography (SEC) with multiangle laser light scattering (MALLS) was used to determine molecular weight and distribution. The SEC was equipped with a prepacked column (exclusion limit of M_w 1.30×10^6) (Superdex 200 HR 10/30, Amersham Biosciences) and an interferometric refractometer detector (Wyatt/Optilab 903) and a MALLS equipped with a DAWN DSP multiangle laser photometer (Wyatt Technology Co., Santa Barbara, CA) at 25°C. Data was processed by Wyatt Astra software and analyzed by the Debye method. Infrared spectra were obtained on a Nicolet FT-IR spectrophotometer using a KBr disk containing 1% finely ground samples. Viscosities were measured with a capillary viscometer at 25°C. Kinetic energy correction was neglected and a Huggins plot was used to obtain intrinsic viscosity $[\eta]$.

Broiler Performance and Attachment of *Salmonella enteritidis*

A total of 108 day-old broiler chicks were assigned to one of six treatments (3 pens/treatment with 6 birds/pen). The treatments consisted of three diets: control, antibiotic (salinomycin 0.045% of the diet), and corn hull arabinoxylan (0.5% of the diet), and two temperatures (23 and 29°C). Birds were placed on treatment diets on day one and grown for three weeks. Battery cage temperatures were initially set at 35°C and were decreased daily to 23°C and 29°C, respectively, and remained at these temperatures for the remainder of the study. Birds had ad libitum access to food and water.

Broiler weight gain was measured weekly. At the end of three weeks, 20 birds (eight birds from the control and arabinoxylan treatments and four birds from the antibiotic treatment at both temperatures) were killed and intestinal samples were obtained. Two 10-cm sections of the ileum were obtained from each bird

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for organ challenge culture. The ileal sections were flushed with PBS, one end sealed, and 1 mL of kanamycin resistant *Salmonella enteritidis* NAVEL 7759 (provided by Tom Stable, NADC, Ames, IA) at $8.7 \log_{10}$ colony forming units (CFU)/mL was added to the ileal section, and the open end was closed. The intestinal sections were incubated for 1 hr in Dulbecco's modified Eagle's medium (DMEM) at 37°C in a rotary water bath under a 20% CO_2 atmosphere. The ileal sections were then rinsed with PBS, homogenized, serially diluted in peptone broth, and plated on LB agar containing kanamycin. Colonies on plates were counted and colony numbers were converted to \log_{10} CFU/g of wet tissue.

Macrophage Activation Assay

A murine macrophage cell line RAW 264.7 (T1B-71, American Type Culture Collection [ATCC], Rockville, MD) was provided by Bruce Watkins, Department of Food Science at Purdue University. This cell line was grown for two to three days in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere supplied with 7% carbon dioxide. Viability of cells used in experiments was always $>90\%$ as determined by the trypan blue exclusion test. Cell monolayers were seeded onto 96-well tissue culture plates and cultivated for three days to 7.3×10^4 cells/well. Then 90 μL of phosphate buffered saline (20 mM PBS 7.2) and 10 μL of hemicellulose solutions were introduced into preparative 96-well microplates to final hemicellulose concentrations of 100, 50, and 25 $\mu\text{g}/\text{mL}$, while an equal volume of phorbol 12-myristate 13-acetate (PMA, Sigma P-8139) was added to selected wells at 0.2 $\mu\text{g}/\text{mL}$ and *Salmonella typhimurium* lipo-

polysaccharides (LPS, Sigma L-9516) at 25 $\mu\text{g}/\text{mL}$ serving as positive controls (Brubacher and Bols 2001; Xie et al 2001). Then 10 μL of DCF-DA (2',7'-dichlorodihydrofluorescein diacetate, Sigma D-6883) at 10 μM concentration was added to each well and an equal volume of PBS without DCF-DA served as the blank (Rath et al 1998). All concentrations were expressed as final contents in each well. RAW cell monolayers were washed three times with PBS, and the tested mixtures in the preparative microplate were transferred to the 96-well tissue culture plates containing RAW cells immediately. Oxidation of the probe DCF-DA was measured at 15-min intervals for 2 hr using a spectrofluorometer (Gemini, SpectraMAX Molecular Devices, Sunnyvale, CA) with excitation and emission wavelength set at 485 and 530 nm, respectively, and the fluorescence was expressed in relative fluorescence units (RFU).

Differences between treatments were analyzed using one-way ANOVA and followed by Tukey's multiple range tests. Values at $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Hemicelluloses

Corn hull arabinoxylan has an extensively substituted backbone of β -(1 \rightarrow 4)-D-xylopyranosyl units with side chains of α -L-arabinofuranosyl units, attached mainly at O-3 positions, but also at the O-2 position of the D-xylose moieties. There may be other groups attached, such as α -D-glucuronic acid residues at O-2 and D-galactose residues (Whistler and Corbett 1955; Saulnier et al 1995). Corn hull arabinoxylans exist as wormlike chains and aggregates in aqueous solution with an apparent MW of 5.06×10^5 (Zhang et al 2003).

Fiber composition of banana peel is $\approx 42\%$, including 22% hemicelluloses, 6.3% cellulose, and 0.6% lignin (Kayisu et al 1981; Whistler 1998). Here, hemicellulose A (water insoluble, yield 2.3%) consists of D-xylose (92%) with L-arabinose (8%). Hemicellulose B (water soluble, yield 9.2%) consists of L-arabinose (52.8%), D-xylose (2.6%), and D-galactose (21.6%) (Table I). FT-IR spectra of hemicellulose A with absorbance at 3423, 1641, 1401, 1163, 1091, 1045, 988, 898 cm^{-1} and hemicellulose B 3446, 1641, 1401, 1320, 1091, 1044, 899 cm^{-1} are presented in Fig. 1. Bands at 898 and 899 cm^{-1} are characteristic of β -glycosidic linkage between the sugar units (Sun et al 1996). The band at 1641 cm^{-1} is due to linked water (Sun et al 1996, 2000). Bands at 3423–3446 cm^{-1} are due to the –OH stretch vibrations of hemicellulose and water involved in hydrogen bonding (Kacurakova et al 2000). Intensity at 1401 cm^{-1} implies C-H bending in hemicellulose A and B. The band at 1163 cm^{-1} is due to ν (C–O–C) of xylose in hemicellulose A (Sun et al 2000). A band at 1321 cm^{-1} is due to –CH₂ deformation of arabinose, which indicates a highly branched arabinose unit. A band at 988 cm^{-1} is due to arabinose residues attached only at the O-3 of xylose residues.

FT-IR data indicate that hemicellulose B appears to be a β -D-xylan with D-xylosyl and L-arabinosyl branches, probably mixed with arabinogalactan, while hemicellulose A is β -D-xylan with L-arabinose branches. Further analyses using methods such as GC-MS and ^{13}C NMR will be made to clarify and confirm chemical structures.

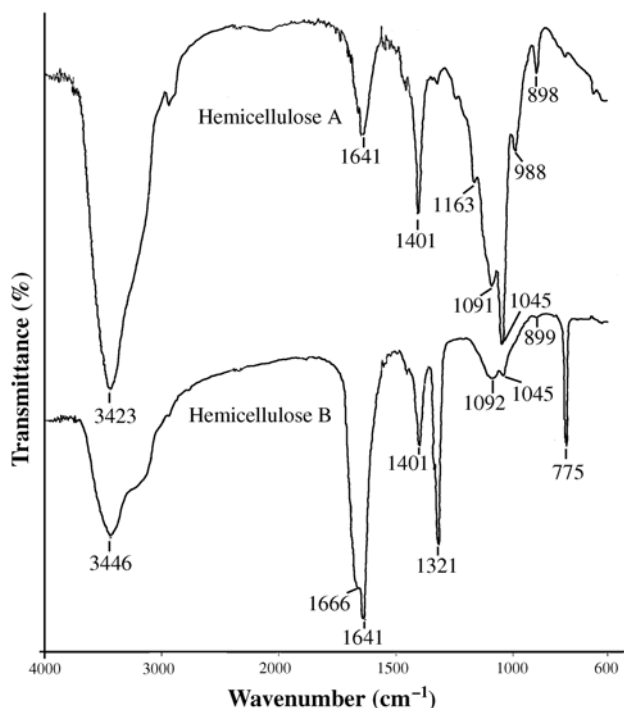


Fig. 1. Infrared spectra of banana peel hemicellulose A and B.

TABLE I
Banana Peel Hemicellulose Extracted with 4% Sodium Hydroxide

Hemicellulose	M_w	Yields (%) ^a	Monosaccharide				$[\eta]$ cm ³ /g
			Arabinose	Xylose	Galactose	Glucose	
A	...	2.3	8.0	92.0	1.5 ^b
B	2.88×10^5	9.2	52.8	25.6	21.6	...	8.4

^a Calculated as a percentage of dry peels.

^b 0.5M NaOH solution.

Effect of Treatment on Broiler Performance and Ileal Attachment of *Salmonella*

Broilers reduced weight gain at high environmental temperatures (Table II). It appeared that there was a temperature effect on bird weights, indicating maintaining birds at 29°C was a mild stressor. Somewhat surprisingly, birds on the growth promotant antibiotic had lower body weight gain than did the control birds. Birds fed arabinoxylan diet at the higher temperature tended to be heavier (average 572 g) than the control birds (average 486 g) at three weeks of age. These data suggest that the arabinoxylan diet had little effect on unstressed birds but tended to increase body weight gain in birds that were undergoing a continuous mild stress. There was little effect of temperature on attachment of *Salmonella* to the ileal lining; however, at the lower temperature there

TABLE II
Bird Body Weight (BW) after Treatment with Arabinoxylan

Temperature	Treatment	BW (g)	
		Initial	Three Weeks Old
23°C	Control	41.2	608.8
	Antibiotic ^a	41.5	567.8
	Arabinoxylan ^b	41.3	542.1
	SEM ^c	1.4	31.4
29°C	Control	41.2	486.4
	Antibiotic	41.0	481.4
	Arabinoxylan	41.1	572.0
	SEM	0.5	54.0

^a Salinomycin 0.045% of the diet.

^b 0.5% of the diet.

^c Standard error of means.

TABLE III
Attachment of *Salmonella* to Chick Ileal Tissue (log₁₀ CFU/g of wet tissue)

Treatment	23°C	29°C
Control	6.25b ^a	6.49
Antibiotic ^b	6.65a	6.70
Arabinoxylan ^c	6.38b	6.33
SEM ^d	0.67	0.21

^a Values followed by the same letter in the same column are not significantly different ($P < 0.05$).

^b Salinomycin 0.045% of the diet.

^c 0.5% of the diet.

^d Standard error of means.

TABLE IV
Rate of DCF-DA Oxidation in Macrophage Cell Line (RAW 264.7) Upon Exposure to BP-HB and CH-AX at 120 min^a

Treatments	Rate of Change (RFU/min) Mean ± SD
PBS (blank)	0.54 ± 0.21a ^b
DCF-DA (background)	1.64 ± 0.09b
LPS (25 µg/mL)	2.04 ± 0.20c
PMA (0.2 µg/mL)	2.77 ± 0.53d
CH-AX (100 µg/mL)	2.84 ± 0.41d
CH-AX (50 µg/mL)	2.34 ± 0.27c
CH-AX (25 µg/mL)	2.30 ± 0.31c
BP-HB (100 µg/mL)	2.42 ± 0.49c
BP-HB (50 µg/mL)	2.13 ± 0.57c
BP-HB (25 µg/mL)	2.01 ± 0.35c
LAG (50 µg/mL)	1.67 ± 0.45b
LAG (25 µg/mL)	1.64 ± 0.23b
OL-HB (50 µg/mL)	1.63 ± 0.15b
OL-HB (25 µg/mL)	1.54 ± 0.13b

^a PBS, phosphate buffer saline; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; CH-AX, corn hull arabinoxylan; BP-HB, banana peel hemicellulose B; LAG, larch arabinogalactan; OL-HB, olive hemicellulose B.

^b Values followed by the same letter are not significantly different ($P < 0.05$).

was a significant increase in *Salmonella* attachment for growth promotant antibiotic-treated birds. Although the differences were not statistically significant at the higher temperature, there was a trend that *Salmonella* attachment was increased in antibiotic treated birds but was reduced in birds fed the arabinoxylan diet (Table III). A 1.5-log increase in attachment has been observed in birds fasted for 24 hr (Burkholder and Patterson, unpublished data), and it may be that the birds needed to be acutely stressed before sampling to see diet effects on attachment of *Salmonella* to the intestinal lining.

In Vitro Macrophage Activation Assay

Immune stimulation was measured by macrophage activation assay. 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) is a fluorogenic probe commonly used in the respiratory burst assay using mononuclear phagocytes (Brubacher and Bols 2001; Xie et al 2001). The proposed mechanism for hemicellulose-induced oxidation of DCF-DA in macrophages is the stimulation of cells with hemicellulose, leading to activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates superoxide to form hydrogen peroxide (H₂O₂). Subsequent oxidation of DCF-DA by H₂O₂ releases DCF resulting in fluorescence by-product.

Macrophages responded with a significant increase in oxidation of DCF-DA in the presence of corn hull arabinoxylan (CH-AX), banana peel hemicellulose B (BP-HB), LPS, and PMA (Fig. 2 and Table IV). PMA appeared to have higher macrophage activation potential than the LPS, which is consistent with the literature (Rath et al 1998). The average rate of DCF-DA oxidation in macrophage cell line upon exposure to BP-HB and CH-AX at 120 min was calculated and appeared to have rates similar to positive control PMA and LPS, however significantly greater than the DCF-DA background (1.64 RFU/min, $P < 0.05$) (Table IV). No significant activation was detected in macrophages when treated with larch arabinogalactan (LAG) and olive hemicellulose B (OL-HB). Time-dependent changes in the oxidation of DCF-DA by macrophage RAW 264.7 in response to treatments of arabinoxylan concentration of 25 µg/mL were shown in Fig. 2, which indicated that oxidation of DCF-DA by macrophages in

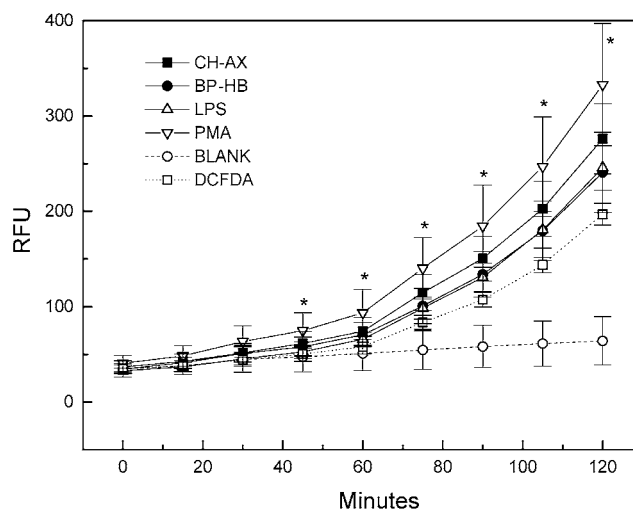


Fig. 2. Time-dependent changes in the oxidation of DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) by macrophages (RAW 264.7) in response to different treatments. Corn hull arabinoxylan (CH-AX) and banana peel hemicellulose B (BP-HB) at concentration of 25 µg/mL, blank (phosphate buffer saline), background (DCF-DA), lipopolysaccharide (LPS 25 µg/mL), phorbol myristate acetate (PMA 0.2 µg/mL). Values were expressed as mean ± SD ($n = 6$ or 12). * Oxidation of DCF-DA by macrophages in response to PMA, LPS, CH-AX, and BP-HB are significantly different from background after 45 min ($P < 0.05$).

response to PMA, LPS, CH-AX, and BP-HB was significantly different from background after 45 min ($P < 0.05$). The results show that hemicelluloses from banana peels and corn hulls enhanced the ability of macrophages to produce metabolites such as reactive oxygen, which indicates activation.

Our preliminary results indicated that arabinoxylan hemicelluloses show positive oxidative burst activity in murine macrophage cells in vitro and tended to increase body weight gain and reduced attachment of the pathogen *Salmonella* to ileal tissue in broiler chicks undergoing mild heat stress in vivo. These data implicate positive beneficial effects of arabinoxylans in cell culture or animal models and therefore, potentially, these would have positive effects on humans. Several polysaccharides were previously tested in cell culture and animal models and subsequently displayed health-promoting effects in humans (Levin 1994; Tzianabos 2000; Coudray et al 2003). An arabinoxylan hemicellulose was recently used for manufacturing a vaccine adjuvant (Fitchett et al 2001). Arabinogalactan-proteins (AGP) are thought to function as immune regulators in human health (Showalter 2001). Water-soluble hemicellulose from soybean hull has also activated macrophages in rats (Nagata et al 2001). Although the activation mode of polysaccharide is not yet established, chemical structure, molecular weight, and appropriate conformations are considered to be important in enhancing activity (Bohn and BeMiller 1995). Further in vivo experiments may ascertain these effects in human syngeneic hosts. All in all, arabinoxylans from the corn hulls and banana peels are biologically active agents and may exert potential health benefits on humans.

CONCLUSIONS

Corn hulls and banana peels represent rich sources of arabinoxylan hemicelluloses. Corn hull arabinoxylan and banana peel hemicellulose B have promoted growth in stressed chicks in vivo and stimulated murine macrophage in vitro. These results indicate arabinoxylans from corn hulls and banana peels have the potential to be used as health-promoting dietary supplements.

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