Scientific Research Publishing

Advances in Enzyme Research, 2025, 13(3), 35-41
https://www.scirp.org/journal/aer
ISSN Online: 2328-4854
ISSN Print: 2328-4846

Combinatorial Enzyme Digestion of Arabinoxylan to Produce Feruloyl Oligosaccharides with Antimicrobial and Antioxidant Activities

Dominic Wing Sum Wong*, Sarah Beth Batt, William John Orts

Western Regional Research Center, USDA-ARS, 800 Buchanan Street, Albany, CA 94710, USA
Email: *Dominic.Wong@usda.gov

How to cite this paper: Wong, D.W.S., Batt, S.B. and Orts, W.J. (2025) Combinatorial Enzyme Digestion of Arabinoxylan to Produce Feruloyl Oligosaccharides with Antimicrobial and Antioxidant Activities. *Advances in Enzyme Research*, **13**, 35-41.
https://doi.org/10.4236/aer.2025.133003

Received: July 4, 2025
Accepted: September 12, 2025
Published: September 15, 2025

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Abstract

The combinatorial enzyme approach was employed to convert pretreated wheat insoluble arabinoxylan oligosaccharides carrying feruloyl substituents. The feruloyl oligos (FOS) were isolated by preparative chromatography, and the active fractions pooled, freeze-dried, and demonstrated to possess both antimicrobial and antioxidant activities. The FOS showed a MIC value of 0.9% (w/v, 35˚C, 24 hr.) suppressing cell growth of the ATCC 8739 *E. coli* test organism. The FOS species was also found simultaneously to possess antioxidant activity. At 1% concentration, the FOS showed 636 ± 7 μM Trolox equivalent antioxidant capacity.

Keywords

Format, Wheat Insoluble Arabinoxylan, Feruloyl Oligosaccharide, Combinatorial Enzyme Digestion, Antimicrobial, Antioxidant

1. Introduction

Combinatorial chemistry has been a major focus of pharmaceutical and biotechnological research in drug discovery and optimization [1]. It has also been proposed for applications in agrisciences [2]-[4]. The basic concept of combinatorial chemistry is the synthesis of a vast population (combinatorial library) of structural variants of a parent molecule. The library is then screened in a high-throughput scheme for the few variants carrying targeted new properties of desirable function/activity. Recently, we have applied the concept of combinatorial chemistry to enzyme-catalyzed hydrolytic conversion of plant fibers to bioactive microfibrils/oligosac-

charides [5]. Plant cell walls contain fibrous polymers that are particularly suitable and useful substrates in this scheme. For example, xylan has a β-1,4-linked xlosyl main chain decorated with several side groups, including phenolic (ferulic acid), acetyl, glucuronyl, and arabinofuranosyl groups [6]. Specific enzymes targeting each side group individually or in various combinations under controlled reaction conditions constitute a combinatorial scheme [5]. The enzymes for specific cleavage of these side groups are available commercially or produced by custom cloning, including feruloyl esterase, acetylxylan esterase, β-glucuronidase, and α-L-arabinofuranosidase. The cleavage of the side groups, their positions on the main chain, and types of linkages would affect the cleavage pattern of the main chain and vice versa.

In our previous investigations, we enzymatically hydrolyzed wheat insoluble arabinoxylan (WIA) and screened for bioactive feruloyl oligosaccharides [7] [8]. The present work describes a preparative scale fractionation of combinatorial enzyme digest of hot water pretreated WIA to recover antimicrobial FOS species also showing antioxidant capacity based on the electron transfer method using Trolox as the standard.

2. Experimental

2.1. Materials

The following were purchased from Megazyme (Wicklow, Ireland): Wheat insoluble arabinoxylan, *Thermotoga maritima* β-D-xylanase ((E-XYNATM, GH10), *Aspegillus niger* α-L-arabinofuranosidase (E-AFASE), and *Clostridium thermocellum* feruloyl esterase (E-FAEZCT). Several recombinant ferulic acid esterases (FAEs) from ruminal metagenomics were developed in this lab [9]. TLC plates were from Analtech (Newark, DE). Culture media and Amberlite XAD-2 resin, and antioxidant assay kit (MAK334) were purchased from Sigma (St. Louis, MO). *E. coli* test organism (ATCC 8739) was obtained from ATCC (Manassas, VA).

2.2. Hot-Water Pretreatment of WIA

In preparation for enzyme digestion, WIA was soaked overnight in water (15 g/28.5 ml) in a stainless-steel reactor tube (1"OD × 4.5"L × 0.65" thickness) with 1" stainless steel swage lock end fittings, followed by autoclaving for 20 min at 121˚C and 21 psi. The pretreated WIA was washed 4x with water and fines were removed [9].

2.3. Enzyme Digestion and Chromatographic Separation

The pretreated WIA was hydrolyzed in a mixture of FAEZCT, AFASE and XynATM in various molar combinations, from 0 to 2 nmole per 100 mg substrate, incubated at 40˚C for 24 hr in water. Details of the protocol have been reported previously [8]. Briefly, a total of 6 reaction times each digesting 1.75 g pretreated WIA were combined after incubation, and the supernatant was collected, filtered, and the enzymes inactivated for 10 min at 100˚C. The final volume of ~75 ml was applied to a packed Amberlite XAD-2 column (bed volume = 295 ml). The loaded

column was washed with 3x column volume of water, and the feruloyl oligosaccharides (FOS) were eluted by 50:50 MeOH/H_2O with a flow rate of 1.5 ml/min.

2.4. Analysis of FOS Fractions

Fractions of 20 ml were collected and analyzed for unsaturation (A320 reading). The FOS-containing fractions were combined, filter-sterilized, concentrated by rotary evaporator, and freeze-dried. This FOS pool was analyzed for total phenolic (ferulic) acid [10], total carbohydrate (phenol sulfuric acid method, [11] [12], and reducing sugar (DNSA method [13]).

2.5. Culture Conditions and Antimicrobial Assay

Test microorganism *E. coli* ATCC8739 (mini-pack glycerol freezer stock) was cultured on an MH agar plate ON at 30˚C. Fresh colonies were cultured in 5 ml MH broth at 35˚C and 220 rpm for ~4 hr. The absorbance at 600 nm was measured, and the culture was diluted with fresh MH broth to a final concentration of 1×10^3 cfu/mL based on a standard curve, which was constructed by plotting the number of colonies (by plate count) vs A600 (of the liquid culture). Details were reported in our previous publication [8].

To assay antimicrobial activity, the FOS pool was added at various known concentrations (0 to 1.20%) to the diluted *E. coli* culture. The culture mixtures were incubated for 24 hr at 35˚C and 220 rpm. Cell growth was measured at A600 and expressed by converting to cfu/mL $\times 10^9$ utilizing the standard curve. The minimum inhibitory concentration (MIC) value is defined as the lowest concentration of an antimicrobial that inhibits the visible growth of the test microorganism (such as ATCC *E. coli* 8739 used in this study) in overnight incubation [14].

2.6. Antioxidant Assay

Total antioxidant capacity (TAC) was measured based on the reduction of Cu^{2+} to Cu^{+} which specifically forms a colored complex with a dye reagent with intensity at 570 nm corresponding to TAC in the sample. The standard curve is constructed by plotting A570 versus concentrations (μM) of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E).

Total antioxidant capacity (in μM) = (A570sample − A570blank)/slope(μM).

3. Results and Discussion

The current study used Amberlite XAD preparative column chromatography to isolate FOS fractions from combinatorial enzyme digestion of hot water pretreated WIA as outlined recently [8] [9] [15]. The lyophilized FOS pool produced a puffy white color material which chemically comprised of 241.00 ± 4.12 nmoles ferulic acid, about one feruloyl side group per 25 xylose units. The average size of the oligosaccharides was ~4 xylose units.

The current study confirms the inhibitory effect of FOS on the growth of the test *E. coli* strain (ATCC 8379). The inhibitory effect increased with concentra-

tion, and a complete suppression of cell growth was achieved at a MIC (minimum inhibitory concentration) value of 0.9% w/v (**Figure 1**).

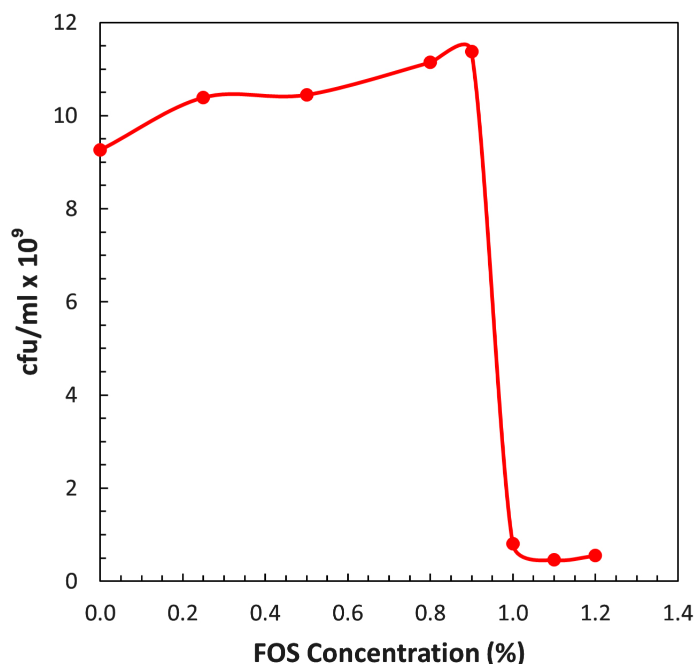

Figure 1. Concentration effects of active FOS species on cell growth by measuring culture absorbance at 600 nm. The results were used to calculate cfm/mL density based on a standard curve.

The antimicrobial activity of FOS was due to the ferulic acid moiety carrying reactive (electrophilic) double bond structures that can participate in inactivation of cell biomolecular reactions. The inhibitory mechanism involves damaging effects on cell wall permeability, disrupting metabolism in cell wall synthesis, and interfering with intracellular enzyme reactions important to cell constituents [16] [17]. In our previous studies [18] on pectic hydrolysate obtained by enzymatic digestion of citrus pectin with endo-polygalacturonase and pectate lyase, we identified active pectic oligo species with antimicrobial properties [18]. The inhibitory action was attributed to the reactive double bonds (formed by the elimination reaction of pectate lyase) the acidic nature of carboxylic side groups, and the small size range of the oligo molecule. In similar studies by others, it has been reported that enzyme digestion of birchwood xylan produces acidic (glucuronic acid-containing) xylo-oligosaccharides, particularly aldopentauronic acids, that are effective inhibitors of certain gram-positive bacteria [19].

The FOS species is expected to possess antioxidant activity, which depends on the hydroxyl and methoxy groups attached to the phenolic acid ring. The existence of the ester bonds in FOS contributes to the high antioxidant activity compared to the free acid [20].

The antioxidant properties of ferulic acids and its possible applications in pharmaceutical and food industry have been reviewed [21]-[23]. In the present study,

using the Trolox equivalent antioxidant capacity (TEAC), is an electron transfer (ET) based method [24]. In this type of reaction, it detects the ability of a potential antioxidant to transfer one electron (reduction of Cu^{2+} to Cu^{+}). The cuprous ion forms a colored complex with a dye reagent disodium [2,2'-biquinolino]4,4-dicarbnoxylate (= BCA bicinchoninic acid sodium salt). The discoloration assay measures the color intensity at 570 nm proportional to the total antioxidant capacity (TAC) of the sample. Based on the Trolox standard curve, the TAC of FOS at 1% was calculated to be 636 ± 7 μM (**Figure 2**).

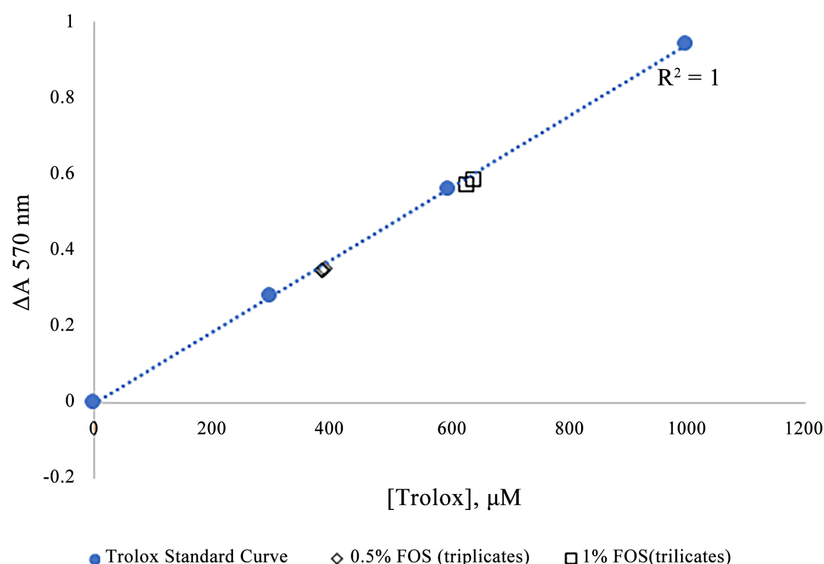

Figure 2. Trolox Standard Curve with inserts of 0.5% and 1.0% FOS A570 points in triplicates.

The production and use of non-digestible oligosaccharides (NDO) has been a thriving industry producing prebiotics for food applications. The health cause-effect of these products is generally linked to the effects on beneficial bacteria in the gut microbiome, due to modification of the physiological environment of the intestinal digestive system [25]. This biological effect of the FOS will be analyzed using metagenomic cell culture studies.

4. Conclusions

Wheat insoluble arabinoxylan was treated by the approach of combinatorial enzyme digestion. Bioactive FOS species in the digest were recovered by preparative chromatographic fractionation. Antimicrobial and antioxidant activities were detected and analyzed. This study revealed the dual-function health benefits of FOS.

Acknowledgements

Reference to a company and/or products is only for purposes of information and does not imply approval of recommendation of the product to the exclusion of others that may also be suitable. All programs and services of the U.S. Department

of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap. The authors declare that there is no conflict of interest regarding the publication of this paper.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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