Scientific Research Publishing

Advances in Enzyme Research, 2024, 12, 35-43
https://www.scirp.org/journal/aer
ISSN Online: 2328-4854
ISSN Print: 2328-4846

Preparative Fractionation of Feruloyl Oligosaccharides Produced by Combinatorial Enzyme Digestion of Arabinoxylan

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How to cite this paper: Wong, D.W.S., Batt, S. and Orts, W.J. (2024) Preparative Fractionation of Feruloyl Oligosaccharides Produced by Combinatorial Enzyme Digestion of Arabinoxylan. *Advances in Enzyme Research*, **12**, 35-43.
https://doi.org/10.4236/aer.2024.123003

Received: August 4, 2024
Accepted: September 27, 2024
Published: September 30, 2024

Abstract

Pretreated wheat insoluble arabinoxylan was converted to oligosaccharides of structural variants using combinatorial enzyme approach. The digestive products were separated by preparative scale chromatographic Amberlite XAD-2 column. Fractions containing feruloyl oligosaccharides (FOS) were isolated, pooled, freeze-dried, and demonstrated to possess antimicrobial activity. The FOS suppressed cell growth of the test organism ATCC 8739 *E. coli* with a MIC value of 0.028% (w/v, 35˚C, 24 hr). The antimicrobial action was observed exceeding 72 hr of culture incubation. The FOS product could be a useful source of prebiotics or preservatives. The present results further confirm the science and application of the concept of combinatorial enzyme technique.

Keywords

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

1. Introduction

The basic concept of combinatorial chemistry is the synthesis of a vast population (combinatorial library) of structural variants of a parent molecular. The library is then screened in a high-throughput scheme for the few variants carrying targeted new properties of desirable function/activity. For example, a simple organic molecule with a core structure of 4 scaffolds (R_1, R_2, R_3, R_4) each carrying randomly arranged 4 types of substituents (W, X, Y, Z) would produce a combinatorial library of $4^4 = 256$ structural variants. Likewise, a hexapeptide randomized with the 20 amino acids at each position would produce a combinatorial library of $20^6 = 64 \times 10^6$ distinct peptide sequences.

The concept of combinatorial chemistry has been a major focus of pharmaceutical and biotechnological research in drug discovery and optimization [1]. It is also useful for applications in agrisciences [2] [3]. Recently, we have applied the concept of combinatorial chemistry to enzyme technology for hydrolytic conversion of plant fibers to bioactive oligosaccharides [4]. Plant cell wall polymers are particularly suitable and useful substrates in this aspect. For example, xylan has a β-1,4-linked main chain decorated with several side groups, including phenolic (ferulic acid), acetyl, glucuronyl, and arabinofuranosyl groups [5]. Specific enzymes targeting each side group individually or in various combinations under controlled reaction conditions constitute a combinatorial scheme [4]. 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 preliminary investigations, we enzymatically hydrolyzed wheat insoluble arabinoxylan and screened for bioactive feruloyl oligosaccharides [6] [7]. The present work describes a preparative scale fractionation of combinatorial enzyme digest of hot water pretreated wheat insoluble arabinoxylan (WIA) to recover FOS species with bioactive property.

2. Experimental

2.1. Materials

Wheat insoluble arabinoxylan obtained from Megazyme (Wicklow, Ireland) was pretreated before using as the substrate. β-D-xylanase from *Thermotoga maritima* ((E-XYNATM, GH10), α-L-arabinofuranosidase from *Aspergillus niger* (E-AFASE), feruloyl esterase from *Clostridium thermocellum* (E-FAEZCT) were obtained from Megazyme. Various recombinant FAEs from ruminal metagenomics were developed in this lab [8]. Test organism ATCC 8739 *E. coli* was obtained from ATCC (Manassas VA). Culture media and Amberlite XAD-2 resin were purchased from Sigma (St. Louis, MO). A glass chromatographic column (5 × 30 cm) was purchased from Bio-Rad (Hercules, CA). TLC plates were from Analtech (Newark, DE). All chemicals are of analytical or HPLC grade.

2.2. Pretreatment of WIA

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 Swagelok end fittings, followed by autoclaving for 20 min at 121˚C and 21 psi. The pretreated WIA was washed 4× with water and fines were removed in preparation for enzyme digestion [9].

2.3. Enzyme Digestion and Chromatographic Separation

A cocktail of FAEZCT, AFASE and XynATM was added to pretreated WIA in

various molar combinations, from 0 to 2 nmole per 100 mg substrate. The reactions were performed in water incubated at 40˚C for 24 hr. 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 3× 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. Fractions of 20 ml were collected and analyzed for unsaturation (A320 reading), total carbohydrate (phenol sulfuric acid method [10] [11], and reducing sugar (DNSA method [12]). The FOS-containing fractions were combined, filter-sterilized, and concentrated by rotary evaporator, and free-dried. This FOS pool was analyzed for total phenolic (ferulic) acid [13], total carbohydrate, and reducing sugar.

2.4. Culture Conditions and Antimicrobial Assay

Test microorganism *E. coli* ATCC8739 (mini-pack glycerol freezer stock) was streaked on an MH agar plate and incubated ON at 30˚C. Five fresh colonies were inoculated in 5 ml MH broth and cultured at 35˚C and 220 rpm. After 4 hr incubation, the absorbance at 600 nm was measured, and the culture was diluted with MH broth to a final concentration of 1×10^3 cfu/ml based on a standard curve. The standard curve was constructed by plotting the number of colonies (by plate count) vs OD600 (of the liquid culture).

To assay antimicrobial activity, the FOS pool was added at various known concentrations (0 to 0.036%) 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 OD600 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 a test microorganism (such as ATCC *E. coli* 8739) in overnight incubation [14].

3. Results and Discussion

In our previous study (Wong *et al.* 2021) it has been shown that hot water pretreatment of corn fiber increased the accessibility to enzyme hydrolysis and 4-fold recovery of ferulic acids. Hot water treatment of lignocellulosic biomass materials has been known to effect dissolution of carbohydrates without causing degradations of the products [15].

The current study used Amberlite XAD, a polymeric adsorbent which has found applications in recovery of phenolic and aromatic compounds (Dow Product Data Sheet 177-02319-0614). The scale-up column showed the ability of considerable clean isolation of FOS from the enzyme digest. The FOS-containing fractions (#44-56, **Figure 1** chromatogram) consisted of increasing phenolic (ferulic) acids and total carbohydrates and decreasing amount of reducing sugars. The FOS pool was freeze-dried to yield a light puffy white powder (**Figure 2**).

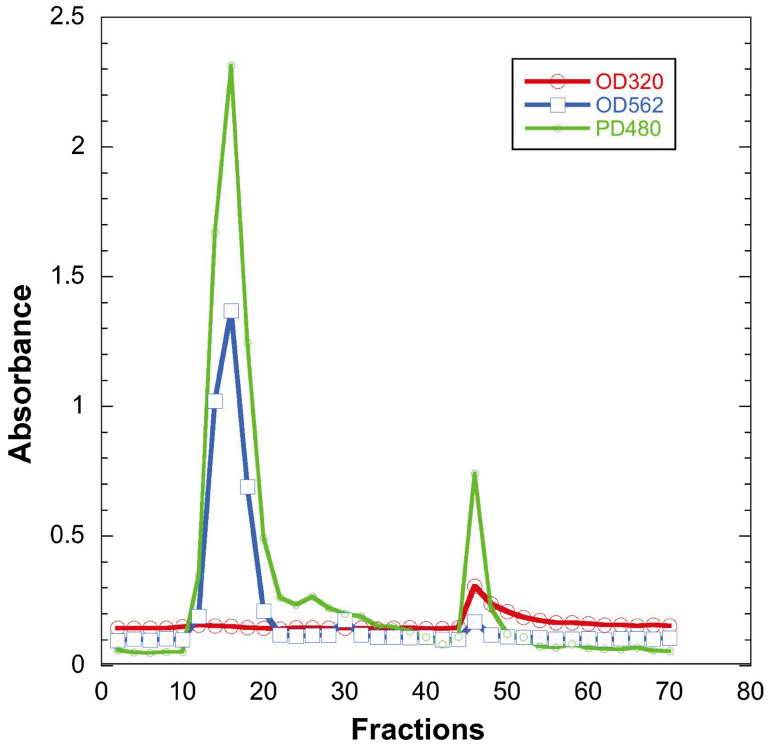

Figure 1. Fractionation of combinatorial enzyme digest of pretreated WIA by preparative scale sorbent chromatography (295 ml bed volume, Amberlite XAD-2). Sample application, washing and elution, see scheme described in "Experimental" section.

Figure 2. Freeze-dried water solution of feruloyl oligosaccharide active species.

The FOS showed an inhibitory effect on the growth of the *E. coli* strain ATCC 8379. In each set of the experiments, the initial inoculation of the microorganism was controlled to 1×10^3 cfu/ml titer, so that the results were comparable based on the same starting conditions. **Figure 3** shows that the inhibitory effect increased with the concentration, and a complete suppression of cell growth was achieved at 0.028% w/v, which was the MIC (minimum inhibitory concentration) value. The antimicrobial effect was sustainable for three days of culturing and

possibly longer (Figure 4).

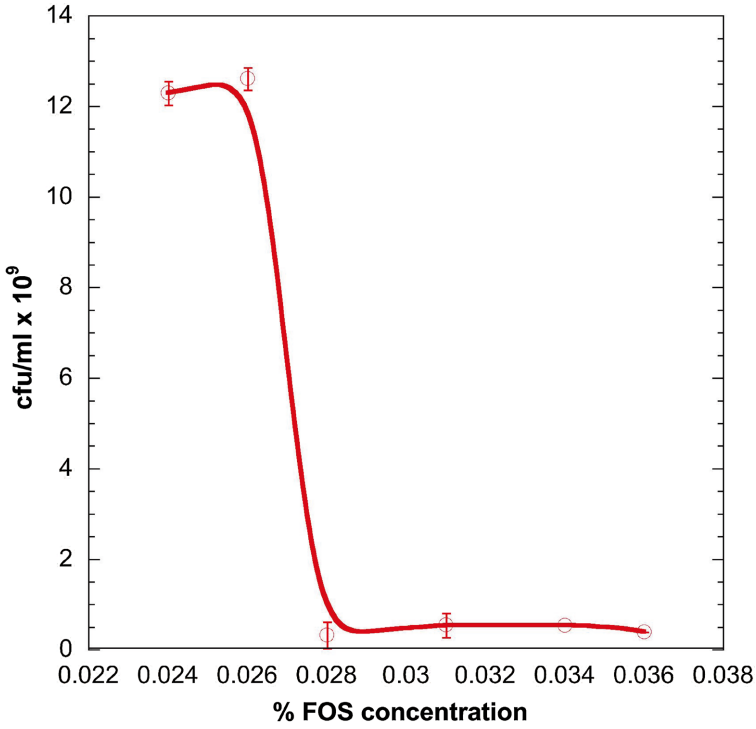

Figure 3. Concentration effects of active FOS species on cell growth. Cell growth was determined by measuring culture absorbance at 600 nm. The results were used to calculated cfm/ml density based on a standard curve.

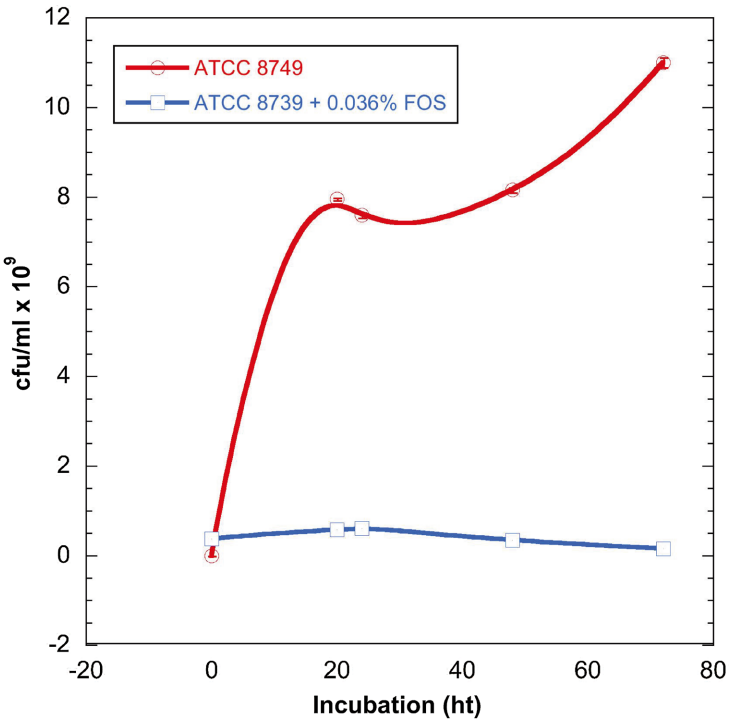

Figure 4. Time course effect of active FOS species added at 0.036% w/v. Control was w/o FOS addition.

The pooled FOS showed an average of 1 ferulic acid moiety per 10 xylosyl residues, composed of an averaging 3.2 xylose units per oligosaccharide. The presence of phenolic acid carrying reactive double bonds was a contributing factor to the antimicrobial effect on cell growth. Double bonds are electrophilic and can participate in crosslinking and inactivation of biomolecules. Phenolic compounds formed in the hydrolysis of lignocellulosic biomass have been shown to exhibit antimicrobial activities [16]. The inhibitory mechanism involves the ability to damage cell walls affecting membrane permeability, disrupt metabolism in cell wall synthesis, and interfere with intracellular enzyme reactions important to cell constituents [16] [17].

The size of the oligosaccharide may be important to facilitating its passage through the cell membrane. High molecular weight oligosaccharides cannot be involved in efficient *in vivo* utilization and contain little biological activities. Various types of antimicrobial oligosaccharides studied and reported generally refer to low molecular weight molecules, consisting of tri-, tetra-, and pentamers [18] [19]. Our previous studies on Bio-gel fractionation of WIA hydrolysate identified the bioactive species with an average size of 1.5 kD [7]. This size range is generally considered in the category of low molecular weight oligosaccharides. It should be noted that the pooled active FOS species most likely represent a mixture of oligo species with similar sizes reflecting the same exclusion pattern on the column matrix. Ishii and Saka [20] prepared a feruloylated arabinoxylan trisaccharide from cell walls of bamboo shoots that inhibits axin-stimulated cell growth of rice plants. The study also indicates that the feruloyl substituent is necessary for the inhibitory effect, and the glycosyl portion is also important for exerting its full activity.

Other functional oligosaccharides have been identified in our previous studies [21]. Pectic hydrolysate obtained by enzymatic digestion of citrus pectin with endo-polygalacturonase and pectate lyase, produced active pectic oligo species with antimicrobial properties [7] [17]. The inhibitory action may be 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, it has been reported 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]. Alginate oligosaccharides consisting of guluronic and mannuronic acids in the main chain, have been shown to potentiate selected antibiotic actions against *Candida* and *Aspergillus* cell growth [22] [23]. Further investigation will be necessary to characterize the structure and function connectivity as well as health cause-effects of these functional oligosaccharides.

The activity of FOS species in the present study is comparable to those of food preservatives generally with a usage of 0.1% range. The production and use of non-digestible oligosaccharides (NDO) has been a thriving industry producing prebiotics for food applications. In recent years, Functional oligosaccharides have been promoted as alternatives for antibiotics in animal production [24]. These

products are also known as natural antimicrobial growth promoters (AGP). 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]. In practical applications, these oligosaccharides are used in sub-minimum concentrations acting to modulate the microbiota condition.

4. Conclusions

1) Libraries of oligosaccharides were generated by combinatorial enzyme digestion of hot water treated wheat insoluble arabinoxylan.

2) Bioactive FOS species were recovered by preparative scale chromatographic fractionation.

3) Antimicrobial activity was analyzed, and the freeze-dried product was obtained.

4) Other bioactive properties such as antioxidant capacity, digestive and immunological effects are in the work.

Acknowledgements

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Conflicts of Interest

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

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