Combinatorial Enzyme Approach for Production and Screening of Libraries of Feruloyl Oligosaccharides

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Abstract

Combinatorial chemistry involves the chemical or biological synthesis of diverse variation of the structures of a target molecule and the library is then screened for variants of desirable target properties. The approach has been a focus of research activity in drug discovery and biotechnology. This report is to demonstrate the application of enzyme technology using the concept of combinatorial chemistry as a novel approach for the bioconversion of plant fibers. Wheat insoluble fiber was subjected to combinatorial enzyme digestion to create structural variants of feruloyl oligosaccharides (FOS). Fractionation and screening resulted in the isolation of a fraction of bioactive FOS species showing antimicrobial activity. These results demonstrate the feasibility and usefulness of the combinatorial enzyme technique in the transformation of plant biomass to value-added products.

1. Introduction

Combinatorial chemistry involves the synthesis of a large array of diverse structural variations of the molecule of interest and the constructed library is then screened for target variants exhibiting desirable activities and functions [1] [2] [3]. A simple organic molecule with a core structure carrying 3 scaffolds, each randomly comprised of 4 types of substituents would result in a combinatorial library of $4^3 = 64$ structural variants. A hexapeptide library randomized with the 20 natural amino acids at each position would produce a population of $20^6 = 64$

million distinct peptides. Chemical techniques used for creating combinatorial libraries involve solid-phase synthesis and its refinements for libraries of small organic molecules, peptides, and oligonucleotides. In contrast, the biological approach uses the genetic code as a precursor to express randomized libraries in microorganisms, such as phage, *E. coli*, and yeast, through systematic and repetitive selection, mutation and amplification. The recent development of dynamic combinatorial chemistry enables the design of "smart" materials libraries of molecules evolved under thermodynamic control by responding and re-equilibrating to external conditions [4].

Numerous reviews have discussed the technology and application of combinatorial chemistry for drug discovery and optimization [3] [5]. Receiving much less attention is the potential use of combinatorial chemistry concept in agrosciences [6], and in agriculture and food research [1] [2]. Plant cell wall polysaccharides, such as xylan, pectin, and xyloglucan, consist of polymeric backbones decorated with various side groups that are cleavage targets of specific enzymes. The presence of these side groups as well as their positions, density, and types of linkages further influence the pattern of enzymatic degradation of the main chain polymer and vice versa. Alteration of the degradation pattern of the modified main chain would in turn lead to hydrolytic products of diverse structures. Enzymatic removal of the side group moieties individually and/or sequentially, therefore, constitutes a combinatorial design. The structural variation expressed in the oligosaccharide products, will translate into changing reactivity and functional properties. Figure 1 presents schematically a few examples of potential oligosaccharide structures from enzymatic digestion of plant fibers.

The hemicellulose polymer xylan contains a β-1,4-xylosyl main chain decorated with at least four types of side groups viz acetyl groups, phenolic-ferulic acids, glucuronyl residues, and arabinofuranosyl residues [7]. The cleavage of these side groups requires acetylxylan esterase, feruloyl esterase, β-glucuronidase, α-L-arabinofuranosidase, respectively. These side groups can be removed in a combinatorial scheme by specific enzymes targeting each group individually or in various combinations under different reaction conditions. The present work
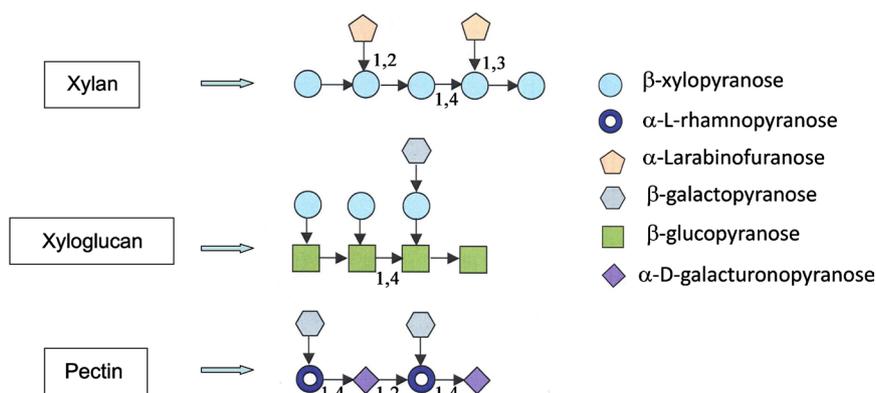

Figure 1. Schematic illustration of some examples of potential oligosaccharide structures from controlled enzymatic hydrolysis of plant fibers.

describes combinatorial enzyme digestion of wheat insoluble arabinoxylan to produce libraries of xylo-oligosaccharides, resulting in isolation of FOS species exhibiting specific functional activities.

2. Materials and Methods

Materials

Wheat insoluble arabinoxylan (WIA), *Aspergillus niger α-L*-arabinofuranosidase (AnABF), *Bifidobacterium adolescentis α-L*-arabinofuranosidase (BaABF), and *Thermotoga maritima β-D*-xylanase (GH10) (TmXYN) were purchased from Megazyme (Wicklow, Ireland). *E. coli* (ATCC 8739) was obtained from American Type Culture Collection (Manassas, VA). Mueller Hinton (MH) broth and Amberlite XAD-2 were obtained from Sigma (St. Louis, MO). Sephadex LH20 was purchased from GE Healthcare (Pittsburg, PA). HPTLC plates were purchased from Analtech (Newark, DE). All chemicals and solvents were of analytical or HPLC grade. Distilled, Milli-Q water was used in all experiments.

Enzymatic hydrolysis of wheat insoluble arabinoxylan

WIA (100 g) was suspended by vortexing in 10 ml water and soaked for 30 min at room temperature. AnABF and/or BaABF of varying nmole concentrations were added to the suspended substrate and mixed well by brief vortexing, followed by the addition of TmXYN at various concentrations. The mixture was incubated at 40˚C for 24 hr with shaking, and centrifuged at 4225 RCF for 40 min. The supernatant recovered was heated at 100˚C for 10 min to inactivate the enzymes and filter sterilized (Nalgene rapid flow filter unit).

Hydrophobic-interaction chromatography

The enzyme hydrolysate was fractionated at room temperature using an Amberlite XAD-2 column (2.5 × 50 cm). The enzyme digest was first eluted with 470 ml H_2O at a flow rate of 0.7 ml/min. Fractions of 12 ml were collected. After washing with H_2O, 800 ml of CH_3OH/H_2O (1:1 v/v) was used to elute the adsorbed FOS from the column. The fractions were measured for ferulic acids by A_{320} reading, for reducing sugars by the DNSA method [8], and for total carbohydrates by the phenol-sulfuric acid method [9] [10]. Fractions were pooled, concentrated by rotary evaporator, and reconstituted in H_2O or suitable solvent.

Size-exclusion chromatography

The pooled fractions of FOS were dissolved in CH_3OH/H_2O (1:3 v/v), and further purified using a Sephadex LH20 column (2.5 × 70 cm). Separation was performed with 1:3 CH_3OH/H_2O as eluant at a flow rate of 0.6 ml/min. Fractions of 12 ml were collected with absorbance measured at 320 nm. Individual peaks were concentrated for further analysis of ferulic acids by HPLC and carbohydrate content as xylose equivalent by the phenol-sulfuric acid method as described below.

Measurement of xylose equivalent and ferulic acid content

The ferulic acid content in the concentrated samples was quantified by HPLC after alkaline hydrolysis (1N NaOH, 37˚C, 16 hr) [11]. The carbohydrate con-

centration as xylose equivalent was determined by the phenol-sulfuric acid method based on a xylose standard curve [9] [10].

HPLC analysis

HPLC analysis was performed on a Shimadzu LC-10 AD system fitted with a UV and RI detectors connected in tandem, using an oligosaccharide column (Supelcogel Ag+) (4.6 × 250 mm) with H_2O as the mobile phase at a flow rate of 0.3 ml/min at 85°C. Gel permeation chromatography (GPC) was performed using an Ultrahydrogel column (7.8 × 300 mm), with water as the mobile phase at a flow rate of 0.3 ml/min. Pullulan standards were used for molecular weight calibration. Analysis of ferulic acid concentration was performed on a Gilson system with a UV detector, using a Brownlee analytical C18 5μ ODS column (260 × 4.6 mm), with H_2O/HCOOH/CH_3CN 7:1:2 mobile phase at a flow rate of 0.2 ml/min.

High-performance thin-layer chromatography

The oligo samples were applied to a 20 × 10 cm HPTLC silica gel F254 plate and developed with EtOAc/HOAc/H_2O (2:2:1). The plate was then sprayed with 10% H_2SO_4 in methanol containing 1 mg/ml orcinol, followed by heating at 90°C for visualization of oligosaccharides.

Growth experiments and culture conditions

A freeze-dried *E. coli* (ATCC 8739) was rehydrated with 1 ml sterile Difco™ MH (Mueller Hinton) broth and streaked onto a MH agar plate. A 5 ml culture (35°C, 210 rpm, 4 hr) was prepared and the absorbance at 600 nm was measured. 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 (liquid culture). The active FOS species (filter sterilized) was added to the diluted *E. coli* culture at various concentrations. Incubation was conducted at 35°C, 210 rpm for various time periods. Cell growth was determined by measuring the absorbance at 600 nm of appropriate dilutions of the cell culture (SpectraMax M2, Molecular Devices, CA) and confirmed by plate counts. The oligo concentration as xylose equivalent was determined by the phenol-sulfuric acid method based on xylose standard curve. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation [12] [13].

3. Results and Discussion

Feruloyl oligosaccharides have been studied using various degradation methods, including acid hydrolysis, hydrothermal processing, microwave-assisted autohydrolysis, and enzyme treatment of plant fibers such as corn and wheat bran, sugar-beet pulp, and insoluble wheat arabinoxylan [14] [15] [16] [17]. Most of these methods involved non-specific hydrolytic breakdown of the fiber and risked the uncontrolled formation of side products. Enzyme treatments are mostly restricted to the use of endo-xylanases, producing oligosaccharide products of limited structural variations. The present work employs a combinatorial

concept utilizing the interactive effects of three enzymes viz *Thermotoga maritima* *β-D*-xylanase (TmXYN), *Aspergillus niger α-L*-arabinofuranosidase (AnABF), and *Bifidobacterium adolescentis α-L*-arabinofuranosidase (BaABF). The *T. maritima* xylanase is a GH10 family xylanase which has an active site that can accommodate xylose residues decorated with side chains, producing small size oligosaccharide products, in contrast to GH11 xylanases that show preference for unsubstituted regions in the xylan backbone [18]. The two *α-L*-arabinofuranosidases AnABF and BaABF are members of specific classes of ABF-m and ABF-d3, respectively. ABF-m removes mono-substituted arabinofuranosyl (Araf) residues from arabinoxylans, whereas ABF-d3 removes the *O*-3 Araf from *O*-2,3-disubsituted Xyl*p* in arabinoxylans [19]. The action of each of these enzymes interactively affects the cleavage patterns among each other and the final outcome of the products, resulting in an expanded diversity of structural variation [20].

The soluble supernatant obtained from combinatorial enzyme digestion of wheat insoluble arabinoxylan was fractionated to obtain ferulic acid-containing oligosaccharides (FOS) (Figure 2). In contrast to the other fractions, the FOS fractions consisted of phenolic acid moieties as indicated by absorbance at 320 nm. From the elution pattern, the FOS fractions were relatively more hydrophobic than the rest. The FOS yield (fractions 37 - 46) was about 10 - 13 percent based on xylose-equivalent, containing 98% of the ferulic acid content in the WIA digest. The ratio of xylose:FA was in the range of ~55:1 based on the xylose equivalent (by the phenol-sulfuric acid method) and FA content (by HPLC analysis of alkaline hydrolyzed sample) as described in the "Methods" section. The FOS was applied to a Sephadex LH20 column, which further separated into
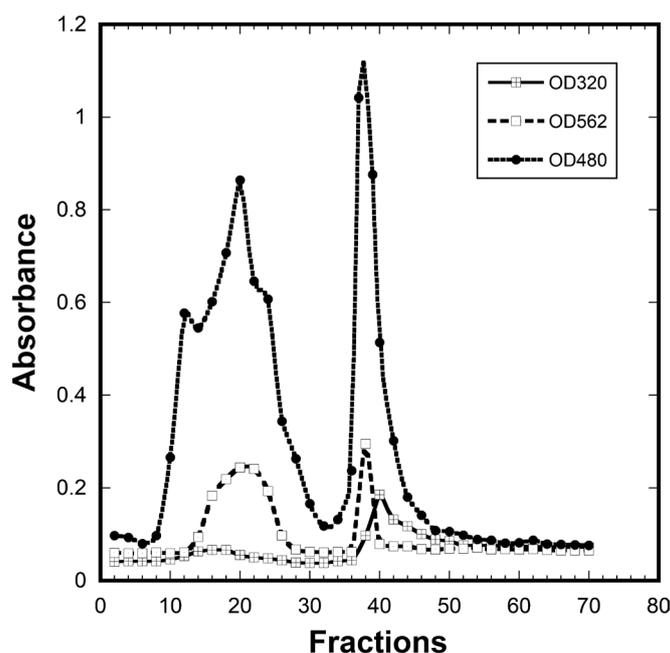

Figure 2. Hydrophobic chromatographic fractionation of enzyme-digested WIA. Column: 2.5 × 50 cm, Amberlite XAD-2; Flow rate: 0.7 ml/min; Stepwise elution: 450 H_2O, 800 ml CH_3OH/H_2O (1:1 v/v), Fractions collected: 12 ml/tube.

a major peak showing absorbance at 320 nm (indicating phenolic acids) and positive with the phenol-H_2SO_4 assay (suggesting carbohydrate neutral sugars).

The FOS showed an inhibitory effect on the growth of the *E. coli* strain ATCC 8379 in an initial testing. The enzyme-derived FOS was screened with the test microorganism *E. coli* ATCC 8379 for bioactive properties. In each set of experiments, the initial inoculation of the microorganism was carefully controlled to 1×10^3 cfu/ml titer, so that the comparisons were performed under same starting conditions. Figure 3(a) shows that the inhibitory effect increased with the concentration, and a complete suppression of cell growth was achieved at 0.07% w/v, which was the MIC (minimum inhibitory concentration) value. The antimicrobial effect was sustainable for three days and possibly longer (Figure 3(b)).

The FOS major peak from the LH20 column was analyzed by HPLC gel permeation chromatography showing a peak of MW $\simeq$ 1.3 kD (based on a pullulan standard curve) (Figure 4). This size range is generally considered in the category of low molecular weight oligosaccharides. The small size of the oligosaccharide may be a factor in facilitating its passage through the cell membrane. Reports of antimicrobial oligosaccharides generally refer to low molecular weight molecules, consisting of tri-, tetra-, and pentamers. Oligosaccharides in the high molecular weight range have been shown to prevent efficient *in vivo* utilization and generally are not effective in functional and biological activities [21] [22]. The present results seem to support the suggestion that small molecular
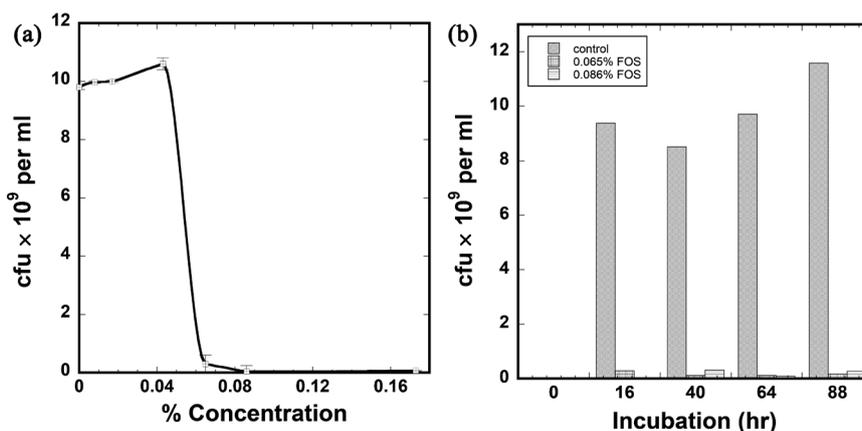

Figure 3. (a) Effect of the active FOS on cell growth. An overnight culture of *E. coli* (ATCC 8739) in MH broth at 35°C was diluted to appropriate titer concentrations, and various concentrations of FOS (0.008% to 0.17%) were added. The incubation was continued for 16 hr. Cell growth was determined by measuring A_{600} of appropriate dilutions of cell culture, and the cfu/ml density was calculated based on the conversion curve. (b) Time course effect of the active DOS on cell growth. An overnight culture of *E. coli* (ATCC 8739) in MH broth at 35°C was diluted to appropriate titer concentrations, and the FOS species at 0.065 and 0.086% was added. The incubation was continued for 4 days with sampling at 16, 40, 64 and 88 hr. Cell growth was determined by measuring A_{600} of appropriate dilutions of the cell culture, and the cfu/ml cell density was calculated using conversion curve. All experiments were performed in duplicates. Vertical bars on the data points in the figure represent standard deviations. In some case where the s.d. is very small compared to the axis scale, the bars may not be clearly visible.
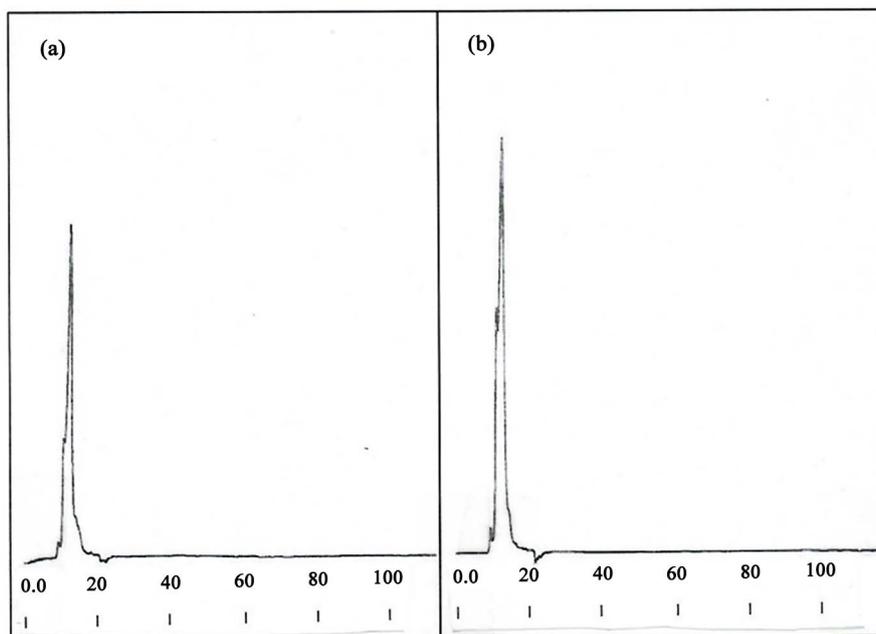
Figure 4. HPLC gel permeation chromatography of the active FA-oligo species. Analysis was performed on a Shimadzu LC-10 AD system fitted with UV-Vis and RI detectors connected in tandem, using a Ultrahydrogel 120 column (Waters, 4.6 × 250 mm). The mobile phase was H_2O run at a flow rate of 0.3 ml/min at room temperature. (a) RI and (b) UV-vis 315 nm detection.

size may assist in the adhesion and penetration of the cell membrane, and increase potential interactions with intracellular constituents and processes. It is noted that the major peak on HPLC may not necessarily represent a single structure, but possibly a mixture of oligo species with similar sizes reflecting the same exclusion pattern on the gel matrix.

The mechanism of inhibition may be linked to the unique structural properties of the active species. The antimicrobial activity of some classes of phenolic compounds has been attributed to the presence of reactive double bonds and the association of the acid moiety [23]. Double bonds are electrophilic and can participate in a variety of reactions, resulting in crosslinking and inactivation of biomolecules. Antimicrobial activities of phenolic compounds are found in hydrolysis of lignocellulosic materials, comparable to the common preservative sodium benzoate [24]. Feruloyl oligosaccharides derived from feruloyl polysaccharides have been shown to have biological activities. A feruloylated arabinoxylan trisaccharide FAXX prepared from cell walls of bamboo shoots inhibits axin-stimulated cell growth of rice plants [25]. The study also indicates that the feruloyl substituent is necessary for the inhibitory effect, but the glycosyl portion of FAXX is also important for exerting full activity. Feruloyl oligosaccharides from corn bran and wheat aleurone have been shown to inhibit sucrase and maltase activities of α-glucosidase and glucose absorption [26]. Wheat bran FOS has shown a higher antioxidant activity than free ferulic acid, and *in vivo* protection against oxidative stress in animal studies [27].

Other functional oligosaccharides have been reported in similar studies. Alginate oligosaccharides, consisting of guluronic and mannuronic acids in the main chain, have been reported to potentiate selected antibiotic actions against *Candida* and *Aspergillus* cell growth [28] [29]. Acidic (glucuronic acid-containing) xylo-oligosaccharides, particularly aldopentauronic acids, are effective inhibitors of certain gram-positive bacteria [21]. The pectic hydrolysate obtained by the digestion of citrus pectin with endo-polygalacturonase and pectate lyase resulted in active pectic oligo species with antimicrobial properties [30]. The mechanism of inhibition may be attributed to the unsaturated structure (reactive double bonds) due to the elimination reaction of pectate lyase, the acidic nature (carboxylic side groups) of the substituents, and the small size range of the molecule. Investigation will be necessary to characterize the structure and function connectivity as well as health cause-effects of functional oligosaccharides using molecular, biochemical, microbiological, and physiological studies.

The activity of FOS species in the present study can be considered comparable to those of some common food preservatives, which are generally applied in the range of 0.1%. The use of non-digestible oligosaccharides (NDO) has become a thriving industry providing various prebiotics for food uses. Another potential use may be found in the animal feed industry. Functional oligosaccharides have been promoted as alternatives for antibiotics in animal production. These so-called natural antimicrobial growth promoters (AGP) have gained increasing attention in recent years [31]. The health cause-effect of these products is generally linked to the promotion of beneficial bacteria in the gut microbiome, and modification of the physiological environment of the intestinal digestive system [32]. In practical applications, functional oligosaccharides are used in sub-minimum inhibitor concentrations acting to modulate the microbiota composition.

4. Conclusion

Active FOS species has been derived from combinatorial enzyme digestion and isolated and shown to inhibit the growth of the *E. coli* test organism ATCC 8739. The MIC value was estimated to be comparable favorably to those reported for other active oligosaccharides and some common food preservatives. The FOS species may find useful application as a new source of high-value prebiotics or as alternatives to antimicrobial growth promoters.

Acknowledgements

Conflicts of Interest

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

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