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Feruloyl Oligosaccharides with Antioxidant Activities against Oxygen Radicals

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Abstract

Wheat insoluble arabinoxylan was converted to oligosaccharides carrying feruloyl substituents using the combinatorial enzyme technology. The feruloyl oligos (FOS) were isolated by preparative chromatography, and the active fractions were pooled, and demonstrated to exhibit both antimicrobial and antioxidant activities. The FOS was shown to possess antioxidant activity against oxygen radicals. The Trolox equivalent antioxidant capacity of FOS determined by oxygen radical antioxidant capacity (ORAC) assay averaged 48.2 mM TE for 0.1% solution. The FOS constituted a product with dual functions on health effects.

Keywords

Wheat Insoluble Arabinoxylan, Feruloyl Oligosaccharide, Combinatorial Enzyme Digestion, Antimicrobial, Oxygen Radical, Antioxidant Activity

1. Introduction

The concept of combinatorial chemistry has been applied to enzyme-catalyzed hydrolysis of plant fibers (for example xylan) generating bioactive microfibrils/oligosaccharides [1] [2]. Plant cell wall xylan contains an α-1,4-linked xylosyl chain backbone decorated with several side groups, including phenolic (ferulic acid), acetyl, glucuronyl, and arabinofuranosyl residues [3]. These enzymes targeting side groups individually or in combinations can be controlled by various reaction conditions creating a combinatorial scheme [1]. The types of side groups, their positions on the main chain, and structural linkages would affect the cleavage pattern of both the main chain and side chain.

In our previous investigations, we described a preparative scale fractionation of the combinatorial enzyme digest of hot water pretreated WIA to recover and study

antimicrobial FOS species and showed antioxidant capacity based on the electron transfer method using Trolox as the standard [4]. In the present report, we employed the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the antioxidant capacity of FOS. It works by measuring the ability of a fluorescent probe, fluorescein, to absorb (oxidized/quenched by) free oxygen radicals (peroxy radical $ROO^{\cdot}$ in this case). Antioxidants suppress this reaction by an oxygen radical quenching mechanism to inhibit the oxidative degradation of the fluorescein signal. The antioxidative effect (activity) is then measured as an increase in fluorescence.

2. Experimental

2.1. Materials

The following enzymes were purchased from Megazyme (Wicklow, Ireland): wheat insoluble arabinoxylan, β-D-xylanase (*Thermotoga maritima* E-XYNATM, GH10), α-L-arabinofuranosidase (*Aspergillus niger* E-AFASE), feruloyl esterase (*Clostridium thermocellum* E-FAEZCT). Several recombinant ferulic acid esterases (FAEs) from ruminal metagenomics were developed in this lab [5]. Culture media and Amberlite XAD-2 resin were purchased from Sigma (St. Louis, MO). *E. coli* test organism (ATCC 8739) was obtained from ATCC (Manassas, VA). ORAC antioxidant assay kit was purchased from Cell Biolabs, Inc. (San Diego, CA).

2.2. Pretreatment of WIA

2.2.1. Enzyme Digestion and Chromatographic Separation

Wheat insoluble arabinoxylan (WIA) was used directly as obtained from the supplier (Megazyme). For enzyme digestion, WIA was pretreated as described in previous report [6]. 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, and incubated at 40˚C for 24 hr in water. Details of the protocol have been reported previously [4] [7]. Briefly, ~75 ml of the enzyme digest was loaded onto a packed Amberlite XAD-2 column, washed with 3x column volume of water, and the feruloyl oligosaccharides (FOS) were eluted by 50:50 MeOH/H_2O.

FOS fraction pools were analyzed for total phenolic (ferulic) acid [8], total carbohydrate [9] [10], and reducing sugar (DNSA [11]). Culture conditions and antimicrobial assay were described in detail in previous reports [4] [12].

2.2.2. Oxygen Radical Absorbance Capacity (ORAC) Assay

The following shows how ORAC works in a general mechanism consisting of several steps [13].

In this method, the compound AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride) is used to generate peroxyl radical. The radical reacts with fluorescein, causing it to become oxidized and lose its fluorescence. If an antioxidant is present in the sample, it will scavenge the free radicals and protect the fluorescein from oxidation, delaying/decreasing the loss of fluorescence. The amount of

fluorescence retained over time is measured and this is used to determine the antioxidant capacity of the sample. Peroxyl radical, which is commonly found in the body, makes the assay biologically relevant.

Trolox is used as a standard (a known antioxidant as a reference in calculations) and ORAC results are often expressed as Trolox equivalents (TE). The ORAC assay can measure both hydrophilic and lipophilic antioxidants, providing a comprehensive assessment of the total antioxidant capacity. The assay is widely used in food science, nutraceutical research, and pharmaceutical development to assess the antioxidant potential of various substances.

Antioxidant standards and sample dilutions were assayed in duplicates. A freshly prepared standard curve was used for the assay. Wells were read with a SpectraMax M3 fluorescent microplate reader (Molecular Devices, LLC, San Jose, CA) at 37˚C, in 5-minute increments for 60 minutes, with an excitation wavelength of 480 nm, an emission wavelength of 520 nm, and a 515 nm cutoff filter.

A physiological condition of oxidative stress is often caused by excessive reactive oxygen species (ROS), which may lead to cellular damage, including DNA, protein, and lipid membranes, resulting in the development of many diseases. The production and use of non-digestible oligosaccharides (NDO) have been applied for food applications with health cause-effects linked to the modification of the physiological environment of the intestinal digestive system [14].

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 and confirmed the inhibitory effect of the FOS on the test microorganism *E. coli* strain ATCC8379 with a MIC value of 0.9% w/v as outlined recently [4] [6] [7]. The lyophilized FOS pool which chemically comprised of 241.00 ± 4.12 nmoles ferulic acid (per mg FOS). The current study again confirms the inhibitory effect of FOS on the growth of the test *E. coli* strain (ATCC 8379) showing a complete suppression of cell growth at a MIC (minimum inhibitory concentration) value of 0.9% w/v (reported in [4]).

The antioxidant properties of ferulic acids and possible applications in pharmaceutical and food industry have been reviewed extensively [15]-[18]. The antioxidant activity of FOS species depends on the hydroxyl and methoxy groups attached to the phenolic acid ring. The ester bonds in FOS contribute additional high antioxidant activity compared to the free acid [17].

The present study measured an important aspect of antioxidant capacity of feruloyl oligosaccharides based on its inhibitory effect on free radicals. The relative fluorescence value (RFU) of several time points (5-minute increments) was measured for various concentrations (0 to 50 mM) of Trolox standard or (0 to 0.008%) of FOS. The ORAC activity assay kinetic curve (relative fluorescence units RFU versus minutes) of Trolox standard is presented in **Figure 1**.

The area under the curve (AUC) was calculated for 8 concentrations of dupli-

cate Trolox standards, ranging from 0 to 50 mM. The area under the curve (AUC) was calculated from the RFU for each Trolox concentration using the regression formula as indicated by the shaded area in Figure 2.

$$AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + \ldots$$

and Net AUC = $AUC_{FOS} - AUC_{Blank}$ (Figure 3).

The Trolox standard conversion curve was created by graphing the Net AUC against the Trolox concentration. The resulting curve (Figure 3) was used to calculate the mM Trolox Equivalents (TE) of samples based on the sample Net AUC. These ORAC values are expressed as mM TE/L of sample or mM TE/ml.

The current analysis detects the ability of the antioxidant to transfer a hydrogen atom to AAPH-generated peroxy radical and the resulting effect on the intensity

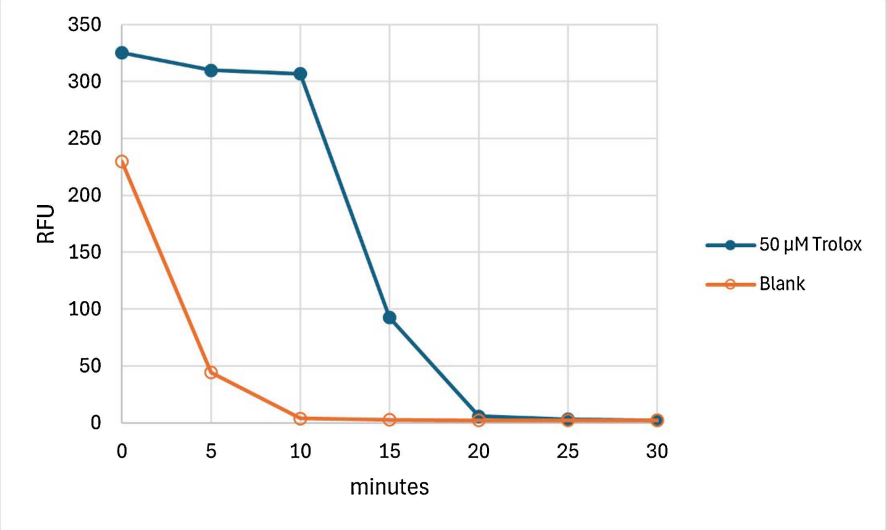

Figure 1. Trolox antioxidant standard ORAC activity assay kinetic curve—RFU versus minutes.

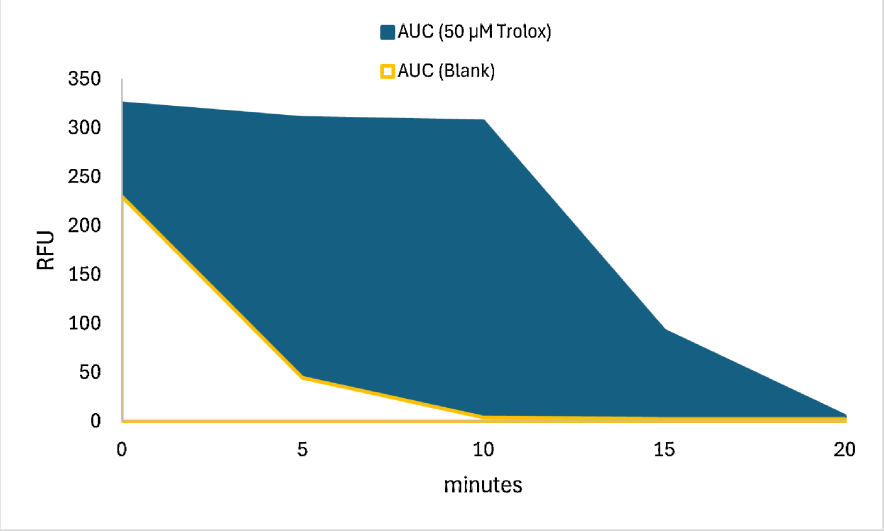

Figure 2. Integrated Trolox antioxidant activity curve (50 mM).

of fluorescein. The decrease in fluorescence intensity can then be expressed as mM Trolox equivalents using a standard curve for calculation. Substitution of Figure 3 equation based on the Trolox Antioxidant standard curve, the Trolox equivalent of each sample was calculated to be an average of 48.18 mM TE for 0.1% solution (0.1 g/100 ml). Figure 4 presents the Net AUC versus FOS concentrations (0.001, 0.002, 0.004, and 0.008%). Corresponding readings from Figure 3 suggest these measurements are within the range of the Trolox Antioxidant Standard curve. The Standard Curve can then be used to calculate Torlox Equivalent (mM TE/L) for the FOS samples.

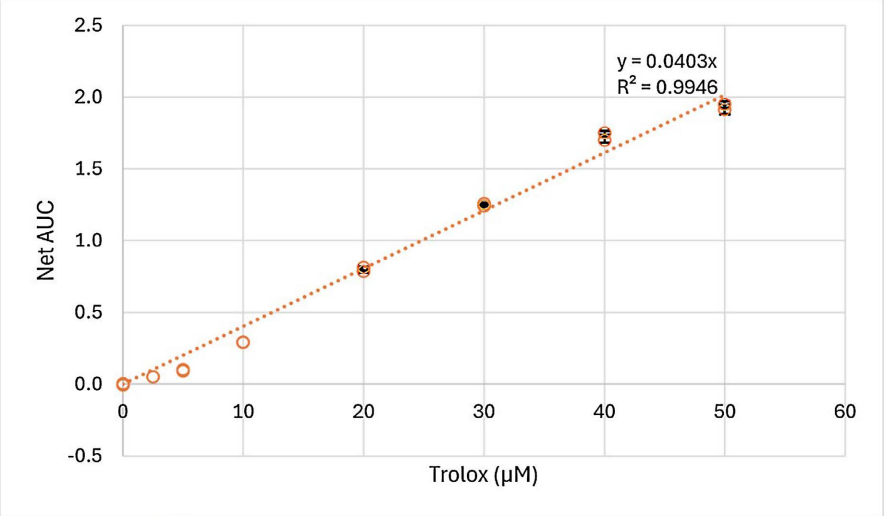

Figure 3. Graphic Net AUC versus Trolox concentration.

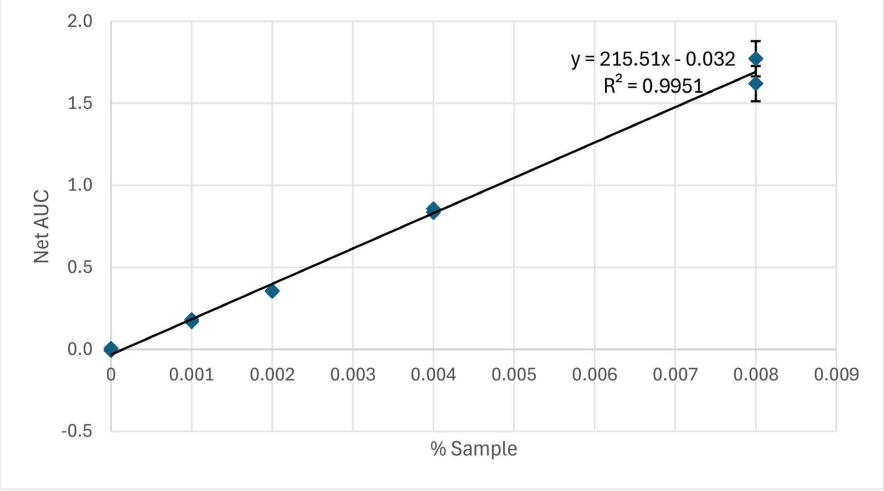

Figure 4. Net AUC versus FOS concentrations (0.001, 0.002, 0.004, 0.008%).

4. Conclusion

Combinatorial enzyme digestion was applied to treat wheat insoluble arabinoxylan to produce libraries of structural variants. Bioactive FOS species in the digest were isolated by preparative chromatographic fractionation. Antioxidant activi-

ties of FOS against ROS were detected and analyzed. Its mode of action was confirmed.

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Reference to a company and/or products is only for purposes of information and does not imply approval or 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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