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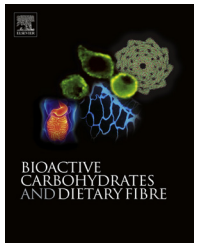

An alternative strategy for structural glucanomics using β-gluco-oligosaccharides from the brown algae *Ecklonia stolonifera* as models

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ABSTRACT

Studies of β-glucans are often hampered by their structural diversity and complexity, which is problematic because interest in their effects on animal cells has increased in recent years. Herein, we present a comprehensive strategy for structural characterization of branched β-glucans, and as a proof-of-concept study, characterized laminarin and acid-soluble β-gluco-oligosaccharides (<4000 Da, void volume elute fraction of gel filtration on Bio-gel P-2) from the brown algae, *Ecklonia stolonifera*. The strategy involves quantitative fluorescence detection-high performance liquid chromatography that enables the characterization of di- and oligosaccharides after acid hydrolysis of the glucan. We found that laminarin is composed of β1–3 (72% in mol) and β1–6 (28%) anomeric bonds, whereas the *E. stolonifera* glucan is composed of β1–3 (57%) and β1–6 (43%) anomeric bonds. This composition is distinct from that of other brown algae β-glucans, for which the β1–6 bond content is much smaller. We also performed a detailed structural analysis of the 11 major β-gluco-oligosaccharides prepared by mild acid hydrolysis and β1–3-specific laminarinase digestion. All 11 oligosaccharides contained branches joined to the backbone by β1–6 bonds. Five of the oligosaccharides had extended branches; in this regard, the *E. stolonifera* glucan is unlike other characterized β-glucans. Our strategy should enable structural characterizations of β-branched glucans, for which no practical approach has been available until now.

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1. Introduction

Glucans are a large group of homopolysaccharides, distributed widely in nature. β-glucans (starch and glycogen) are the most important energy storage substances for both animals and plants. Conversely, β-glucans cannot be universally used as an energy source because only herbivores can digest them. However, β-glucans appear to be recognized as non-self molecules by the innate and adaptive immune systems (Brown & Gordon, 2005) and may, therefore, be effective in the treatment of cancer, including human leukemia (Miyanishi, Iwamoto, Watanabe & Oda, 2003), microbial infects, hypercholesterolemia, and diabetes (Chen & Seviour, 2007; Chan, Chan, & Sze, 2009; Chen & Raymond, 2008). β-glucans, therefore, have the potential to serve as medicinal supplements.

With the exception of cellulose, a linear β1–4 glucan, β-glucans from yeast and fungal cell walls, and from mushroom and brown algae fruiting bodies generally have a β1–3 glucosidic backbone and often contain β1,6 backbone-linked branches (Bull & Chesters, 1966; Chen & Seviour, 2007; Dong, Yao, Yang, & Fan, 2002; Lowman, Ferguson, & Williams, 2003; Manners, Masson, & Patterson, 1973; Manners, Masson, Patterson, Björndal, & Lindberg 1973; Mursito, Jenie, Mubarika, & Kardono, 2010; Schmid et al., 2001). In fact, the major component of the yeast *Saccharomyces cerevisiae* cell wall contains β1–3 bonds in the backbone and a small amount (~3%) of β1–6 backbone-linked branches. Conversely, the β-glucan from the mushroom *Agaricus blazei* contains a backbone with β-1–6 anomeric bonds and one β1–3-linked branch for every three backbone residues. Although many studies have reported partial structures for yeast, fungal, and mushroom β-glucans, few have examined β-glucan (laminarin) from brown algae; e.g., *Laminaria digitata* and *L. hyperborean* (Bull & Chesters, 1966, Hrmova & Fincher, 1993) for which the β1–3-linked backbone dominates.

Ecklonia stolonifera, also a brown algae, inhabits the coast of the Japan Sea from Aomori to Kyusyu, Japan. Although the *E. stolonifera* phycobiont contains β-glucans and β-gluco-oligosaccharides similar in structure to those in *L. digitata* and *L. hyperborean* (Odagiri & Kato, personal communication, 2009), their detailed structure(s) have not been elucidated.

Nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS); (Dong et al., 2002; Ha et al., 2002; Kruppa et al., 2009; Kawai, Igarashi, Yoshida, Kitaoka, & Samejima, 2006; Lowman et al., 2003; Schmid et al., 2001;) have been employed to characterize the *E. stolonifera* β-glucans and β-gluco-oligosaccharides. However, most of these β-glucans have mainly linear structures with relatively short β1–6 branches. Also the use of NMR and GC–MS are laborious, time-consuming, and require a large amount of purified material, which is often difficult to obtain. These difficulties considerably hamper β-glucan and β-gluco-oligosaccharide characterization, which is essential if their structure–function relationships are to be elucidated. Another major difficulty is how to characterize branches (e.g., the β1–3/β1–6 content, and backbone and branch lengths,), which have been assumed to determine the biological activities of β-glucans and β-gluco-oligosaccharides. However, no systematic, highly sensitive procedure had been developed prior to this report, which could be applied to many different types of glucans. Fortunately, all α- and β-anomerically linked disaccharides in glucans are commercially available. Moreover, highly sensitive fluorescent-labeling methods that use monoamine-coupling chemistry (e.g., pyridylamination), have been developed to tag saccharides at their reducing end, thereby providing a method to quantify the glucan content from a biological source and its anomeric bonds when used in conjunction, with fluorescence-detection high-performance liquid chromatography (FD-HPLC).

In this study, we describe a comprehensive procedure to characterize β-gluco-oligosaccharides (<10-mer) that involves: (1) quantitative analysis of the disaccharide components obtained after strong aicd hydrolysis of pyridylaminated purified β-gluco-oligosaccharides by FD-HPLC, (2) structural sequencing of the pyridylaminated β-gluco-oligosaccharides by mild acid hydrolysis, and (3) characterization of the backbone chain length using 1–6-bond-specific acetolysis and laminaridase digestion (Scheme 1). As proof of concept, we extracted β-gluco-oligosaccharides from *E. stolonifera* that contain β1–3 and 1–6 bonds (Odagiri & Kato, personal communication, 2009). The acid-soluble extract contained mainly β-gluco-oligosaccharides that were then characterized using the aforementioned strategy. We found that these oligosaccharides contain a relatively large number of β1–6 bonds (43 mol%), and, as such, represents a new type of brown algae β-glucan. The developed procedure provides a comprehensive strategy for structural characterization of branched β-glucans using a relatively few amounts of samples compared with NMR and GC–MS.

2. Materials and methods

2.1. Materials

Nigerose (Glcα1-3Glc) was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). D-(+)-Maltose monohydrate (Glcα1-4Glc), D-(+)-cellobiose (Glcβ1-4Glc), isomaltose (Glcα1-6Glc), and gentiobiose (Glcβ1-6Glc) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Sophorose (Glcβ1-2Glc) and kojibiose (Glcα1-2Glc) were from SERVA Electrophoresis GmbH (Heidelberg, Germany) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

The brown algae *E. stolonifera* was obtained from Oma town (Aomori, Japan). An Inertsil ODS-3V column ($4.6 \times 250\ mm^2$) and an Inertsil NH2 column ($4.6 \times 150\ mm^2$) were purchased from GL Sciences (Tokyo, Japan). 2-Aminopyridine was purchased from Nacalai Tesque (Kyoto, Japan) and purified from heptane. A Cosmosil 5C18-P column ($2 \times 250\ mm^2$) and a Shodex Asahipak NH2P-50 2D column ($2 \times 150\ mm^2$) were from Nacalai Tesque (Kyoto, Japan) and Showa Denko (Tokyo), respectively. Green coffee bean α-glucosidase and laminarinase from *Trichoderma* sp. were purchased from Sigma-Aldrich (St. Louis, MO), respectively. Laminaribiose (Glcβ1-3Glc), laminaritriose (Glcβ1-3Glcβ1-3Glc), laminaritetraose ($[Glc\beta1\text{-}3Glc]_2$), laminaripentaose ($Glc\beta1\text{–}3[Glc\beta1\text{-}3Glc]_2$), and laminarihexaose ($[Glc\beta1\text{-}3Glc]_3$)