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A Simple Method for Preparing Laminaribiose and Laminaritriose from the Enzymatic Hydrolysates of Curdlan

Naoto ISONO*, Tomomi KIMOTO, and Makoto HISAMATSU
Graduate School of Bioresources, Mie University, Tsu 514-8507, Japan

Abstract

Laminarioligosaccharides (LOS) are used as substrates or inhibitors of several carbohydrate-active enzymes, and exhibit several bioactivities. Commercial LOS are very expensive. In this study, we report a simple method for preparing high-purity laminaribiose (L2) and laminaritriose (L3) from curdlan, which is a linear, high-molecular-weight β-1,3-glucan that is available at low cost. Crude LOS were obtained from the degradation of curdlan by using a commercial enzyme, Zymolyase-20T, prepared from a submerged culture of *Arthrobacter luteus*. Thin-layer chromatography (TLC) analysis showed that the main components of crude LOS were glucose, L2, and L3. In addition, slight amounts of LOS larger than L3 were observed. L2 and L3 were purified from crude LOS by activated carbon chromatography, and their purities were confirmed by TLC and high-performance liquid chromatography (HPLC). Finally, 1.7 g of L2 and 1.2 g of L3 were obtained from 10 g of curdlan.

Key Words: laminaribiose, laminaritriose, laminarioligosaccharides, curdlan, activated carbon chromatography

Introduction

Laminarioligosaccharides (LOS) are oligomers of glucose linked by β-1,3-glucosidic bonds. LOS are used as substrates or inhibitors of hydrolytic enzymes [e.g., β-1,3-glucanase (EC 3.2.1.39), β-1,3-1,4-glucanase (EC 3.2.1.6), and β-1,3-glucosidase (EC 3.2.1.58)] and phosphorolytic enzymes [e.g., laminaribiose phosphorylase (EC 2.4.1.31), β-1,3-oligoglucan phosphorylase (EC 2.4.1.30), and β-1,3-glucan phosphorylase (EC 2.4.1.97)][1]. In addition, LOS have been reported to exhibit significant bioactivities (e.g., anti-apoptotic[2], monocyte-stimulating[3], and prebiotic activities[4]). However, currently, commercial LOS are very expensive [e.g., ＞￥1,000 ($13)/mg for laminaribiose (L2) and laminaritriose (L3)].

LOS can be obtained by hydrolysis of pachyman or curdlan, both of which are linear high-molecular-weight β-1,3-glucans that are insoluble in water[5]. Pachyman is a polysaccharide extracted from the sclerotia of *Poria cocos*[6]. Whelan[7] described a detailed method for the preparation of L2, L3, laminaritetraose (L4), and laminaripentaose (L5) by acid hydrolysis of pachyman and activated carbon chromatography. Curdlan is an extracellular polysaccharide synthesized by the bacteria belonging to the Rhizobiaceae (e.g., *Agrobacterium* and *Rhizobium* spp.)[8]. It is approved as a food additive in several countries, including Japan and USA, and industrially produced at high levels from cheap carbon sources such as by-products of the sugar industry[9]. Recently, Grandpierre et al.[9] reported that enzymatic

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* For correspondence (e-mail: isono@bio.mie-u.ac.jp)

hydrolysis is more favorable than hydrolysis with sulfuric acid or trifluoroacetic acid to produce LOS from curdlan. Glucose, L2, L3, and L4 are the main products formed after hydrolysis with the enzymes prepared from the culture supernatant of the fungus *Trichoderma harzianum*; however, each sugar was not purified from the hydrolysates.

In this study, we developed a simple method for the preparation of sufficient amounts of high-purity L2 and L3, by degrading curdlan using a commercial enzyme, followed by activated carbon chromatography.

Materials and Methods

Carbohydrates

Curdlan was obtained from Takeda-Kirin Foods (Tokyo, Japan). Authentic LOS [currently discontinued; L2, L3, L4, L5, laminarihexaose (L6), and laminariheptaose (L7)] were purchased from Seikagaku Biobusiness (Tokyo, Japan).

Enzymatic degradation of curdlan

Curdlan (10 g) was suspended in 900 mL of water and solubilized by adding 10 mL of 1 M sodium hydroxide. The solution was stirred for 10 min, and then neutralized by adding 50 mL of 1 M Tris buffer (pH 7.0) and 10 mL of 1 M hydrochloric acid. After adding 100 mg of Zymolyase-20T (Nacalai Tesque, Kyoto, Japan) and 1 mL of 1 M dithiothreitol, the mixture was incubated overnight with vigorous stirring at 37 ℃. It was then heated at 80 ℃ for 10 min to inactivate the enzyme, and cooled to room temperature. After centrifugation at 10,000 × *g* for 10 min, the supernatant was collected and used as crude LOS.

Activated carbon chromatography

The crude LOS (~800 mL) were applied to a carbon-Celite [1: 1; powdered activated carbon (Wako Pure Chemical Industries, Osaka, Japan) and Celite-545 (Nacalai Tesque)] column (2.5 × 28 cm) and successively eluted with 1.5 L of 4% (v/v) ethanol and 1 L of 10% (v/v) ethanol. Fractions (20 mL each) were collected at a flow rate of 0.5 mL/min. Finally, fractions containing L2 and L3 were pooled separately, concentrated using a rotary evaporator, and stored at -30 ℃ until use.

Carbohydrate assays

Glucose was determined using the Glucose C2 kit (Wako Pure Chemical Industries). Reducing sugars and total sugars were measured using the 3,5-dinitrosalicylic acid method[10] and the phenol-sulfuric acid method[11], respectively. Glucose was used as a standard in all assays.

Thin-layer chromatography (TLC)

Samples were spotted onto silica gel 60 TLC plates (Merck, Darmstadt, Germany), and developed in ethyl acetate/acetic acid/water (2: 2: 1, v/v/v). The plates were then dried and soaked for 10 s in a solution containing 5% (v/v) sulfuric acid in methanol. After removal of methanol by evaporation, the plates were heated at 110 ℃ for 10 min to visualize the spots.

High-performance liquid chromatography (HPLC)

The final preparations for L2 and L3 were analyzed by HPLC with a refractive index detector. A TSKgel Amide-80 column (5 μm, 4.6 × 250 mm; Tosoh, Tokyo, Japan) was used with acetonitrile/water (65: 35, v/v) at a flow rate of 1.0 mL/min. The column temperature was set at 80 ℃.

Results and Discussion

Enzymatic degradation of curdlan

Curdlan was dissolved in sodium hydroxide solution, and neutralization with hydrochloric acid caused the mixture to become very viscous. However, the viscosity disappeared following overnight digestion with the commercial enzyme Zymolyase-20T, prepared from a submerged culture of *Arthrobacter luteus*. Zymolyase-20T is a mixture of several enzymes, including β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase[12]. TLC showed that the main components of the curdlan hydrolysates (crude LOS) were glucose, L2, and L3 (Fig. 1A). In addition, slight amounts of L4, L5, and larger LOS were observed. The composition of the hydrolysates was similar to that obtained by degradation using the enzymes prepared from a culture supernatant of *T. harzianum*, which contained multiple exo- and endo-β-1,3-glucanases[9].

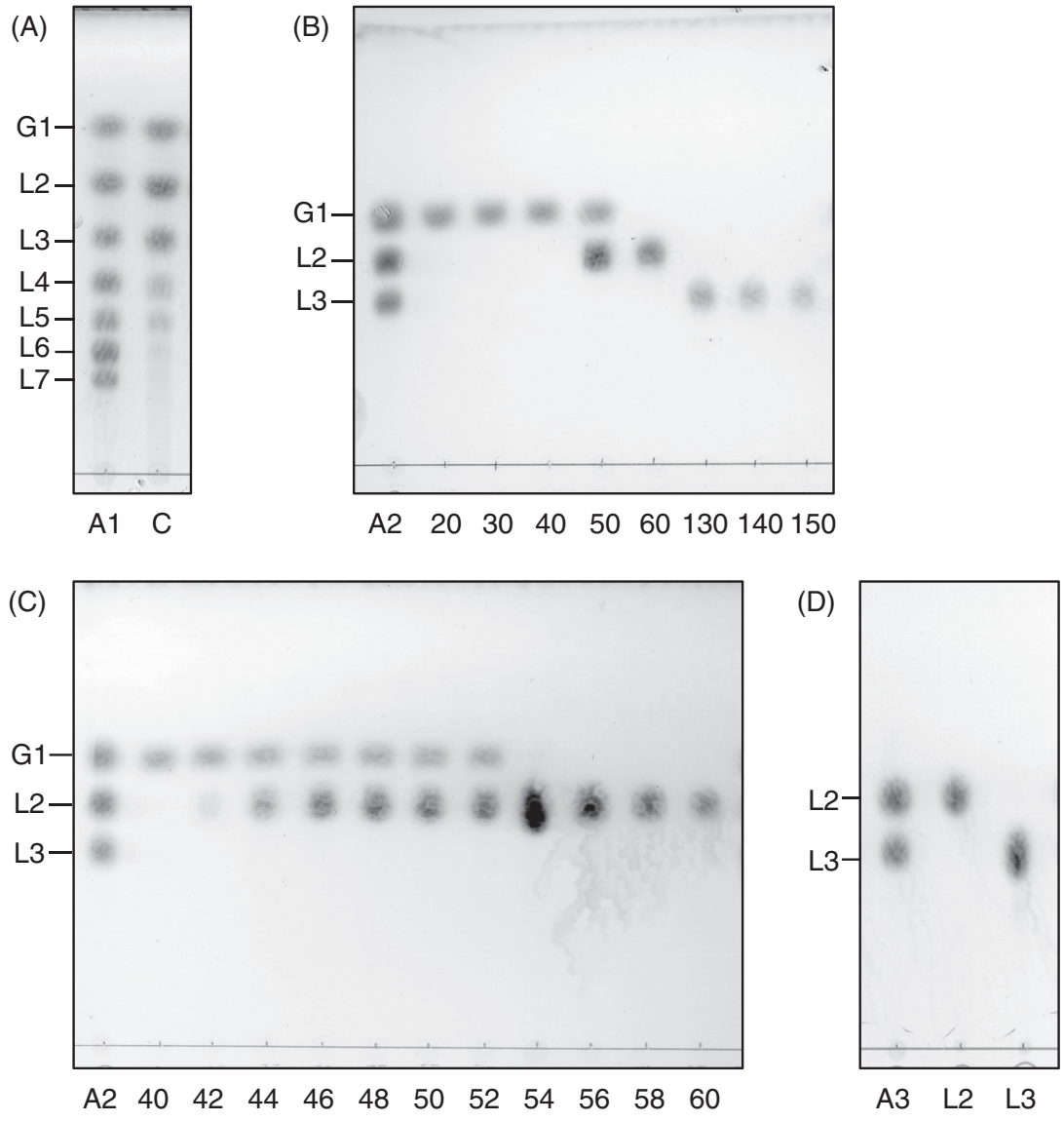

Fig. 1 TLC analysis of LOS.

(A) Crude LOS obtained by the enzymatic hydrolysis of curdlan (twice developed). (B) Eluates from the activated carbon column (fractions 20-60 and 130-150). (C) Eluates from the activated carbon column (fractions 40-60). (D) Final preparations of L2 (34 μg) and L3 (50 μg). G1, glucose; A1, authentic LOS (glucose and L2-L7); A2, authentic LOS (glucose, L2, and L3); A3, authentic LOS (L2 and L3); C, crude LOS (curdlan hydrolysates); numbers refer to fractions.

Purification of L2 and L3 from curdlan hydrolysates

The crude LOS were subjected to activated carbon chromatography to obtain L2 and L3. Quantification of the reducing sugars and glucose as well as TLC analysis was performed on the fractions from the column. Only glucose was detected in the flow-through (fractions 1-40) (Figs. 1B and 2), indicating that activated carbon adsorbed LOS.

Subsequently, 4% ethanol (v/v) was applied to the column to elute L2. Fractions 54-80 contained only L2, while glucose as well as L2 was detected in fractions 42-53 (Figs. 1B, 1C, and 2). In preliminary experiments, we attempted to completely remove glucose from the column by using water or a solution of a composition identical to that of crude LOS, but without curdlan, before the elution of L2. However, these solvents eluted not only glucose, but also a significant amount of L2 for unknown reasons (data not shown). Therefore, the washing step of the column was omitted in this study. Ethanol concentration was

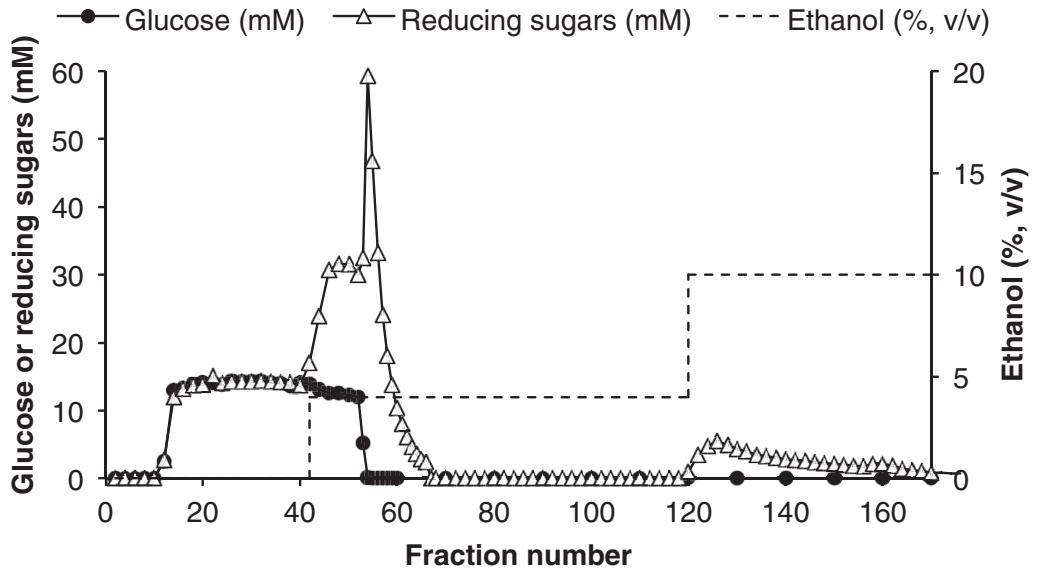

Fig. 2. Elution profile of activated carbon chromatography.

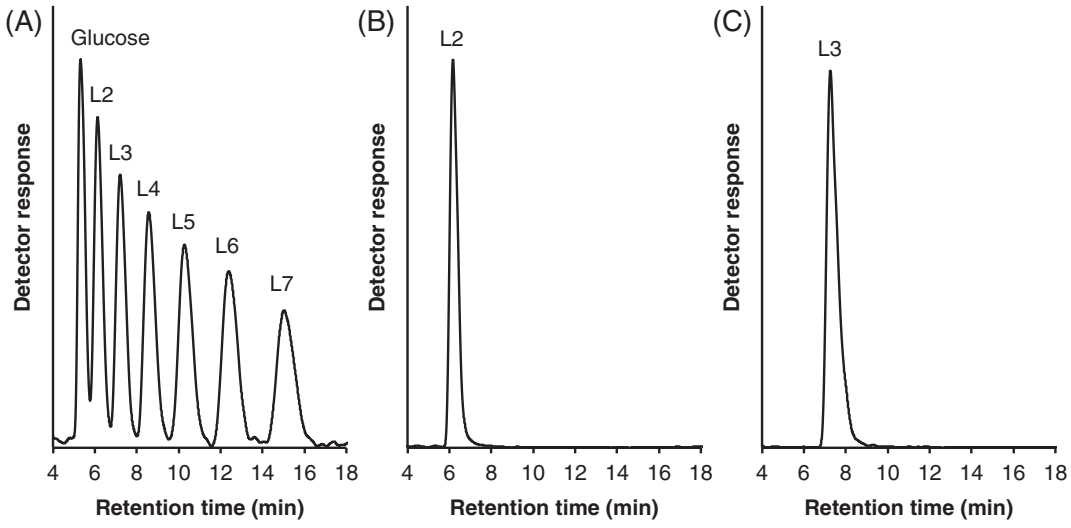

Fig. 3 HPLC analysis of the final preparations of L2 and L3. (A) Authentic LOS (glucose and L2-L7, 50 μg each). (B) Final preparation of L2 (60 μg). (C) Final preparation of L3 (88 μg).

optimized to 4% (v/v) for the complete elution of L2.

L3 was then eluted in fractions 122-170 with 10% ethanol (v/v) (Figs. 1B and2). Larger LOS (e.g., L4 and L5) were not detected in these fractions by TLC analysis.

Fractions 55-72 containing L2 and fractions 122-162 containing L3 were collected and subjected to evaporation to remove ethanol and to concentrate the sugars. Quantification of total sugars showed that 1.7 g of L2 and 1.2 g of L3 were finally obtained from 10 g of curdlan. TLC and HPLC analyses showed that the final preparations of L2 and L3 were of high purity (Figs. 1D and 3). In addition, we confirmed that laminaribiose phosphorylase (EC 2.4.1.31) and β-1,3-oligoglucan phosphorylase (EC 2.4.1.30) from *Euglena gracilis*, as well as β-1,3-glucan phosphorylase (EC 2.4.1.97) from *Ochromonas danica* can use these L2 and L3 preparations as substrates (data not shown).

In this study, we focused on establishing a simple method to prepare L2 and L3 from curdlan. LOS larger than L3 could be produced in sufficient amounts by optimizing the conditions of curdlan degradation (e.g., short-time incubation) and purified using activated carbon column with higher concentrations of ethanol than that used in this study.

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カードランの酵素加水分解によるラミナリビオースとラミナリトリオースの簡易調製

磯野　直人・木本　智美・久松　　眞

三重大学大学院生物資源学研究科

要　　旨

ラミナリオリゴ糖（LOS）は数種類の糖質関連酵素の基質や阻害剤として使用される。また，LOSにはいくつかの生理活性機能があることが知られている。しかし，市販のLOSは非常に高価である。本論文では，高分子の直鎖β-1,3-グルカンである安価なカードランから高純度のラミナリビオース（L2）とラミナリトリオース（L3）を調製する簡単な方法を報告する。*Atrhrobactor luteus* の培養上清から調製された市販酵素Zymolyase-20Tを用いてカードランを分解し，粗LOSを得た。薄層クロマトグラフィー（TLC）分析から粗LOSの主成分はグルコース，L2，L3であることが明らかとなった。また，L3より大きなLOSもわずかに含まれていた。活性炭クロマトグラフィーを用いて粗LOSからL2とL3を精製し，TLCと高速液体クロマトグラフィー（HPLC）により純度を検定した。最終的に，10 gのカードランから1.7 gのL2と1.2 gのL3が得られた。