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A STUDY OF THE LAMINARINS OF SOME FAR-EASTERN, BROWN SEAWEEDS

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ABSTRACT

The structure of laminarins [β-(1→3)-linked D-glucans] from some Far-Eastern, brown seaweeds has been studied. The molecular weights, average number of branch points per molecule, and M- and G-chain contents were determined. The laminarins proved to be similar, differing only in details of fine structure.

INTRODUCTION

β-(1→3)-Linked D-glucans (laminarins) are widely found in micro-organisms and higher plants, where they play the role of structural and reserve polysaccharides[1,2]. The main structural features of laminarins isolated from brown seaweeds have been established, and a current interest is concerned with differences in the fine structure of laminarins isolated from various sources[3–5]. We have undertaken a comparative study of laminarins isolated from Far-Eastern seaweeds.

RESULTS AND DISCUSSION

The laminarins were isolated by treating the seaweed successively with 0.4% hydrochloric acid at room (cold extraction) and elevated temperatures (hot extraction). A partial fractionation of the laminarins with respect to the degree of branching was thus effected. The hydrolysates of the material in the cold and hot extracts usually contained (paper chromatography) glucose, fucose, mannuronic and guluronic acids, and, for *P. wrightii*, galactose also (see Table I).

The mixed neutral and acidic (fucoidin and alginic acid) polysaccharides were separated by using Cetavlon. Glucose was not detected chromatographically in the hydrolysates of the complexes of Cetavlon with the acidic polysaccharides. The laminarins from *Ch. magellanica* and *D. viridis* were subjected to gel fitration on Bio-Gel P30.

The data in Table I show that laminarin was found in seven seaweeds (chromatographic and enzyme-hydrolysis data) but was not detected in extracts from *Sphaerotrihia* sp., *Costaria costata*, and *Chorda fillum*. For further work, five seaweeds (Table I, 1–5) were selected. After separation of acidic polysaccharides and

TABLE I

SOME PROPERTIES OF EXTRACTS FROM FAR-EASTERN BROWN SEAWEEDS

	Seaweed	*Extract*	*Monosaccharide composition*		*Enzymic hydrolysis*[b]	*Yield of pure laminarin (% of wet seaweed)*
			Prior to purification with Cetavlon	*After purification*		
1	*Laminaria cycharioides*	Cold	Glc, Fuc, GulUA, ManUA	Glc	+	4
		Hot	Glc, Fuc, GulUA, ManUA	Glc	+	
2	*Laminaria japonica*	Cold	Glc, Fuc, GulUA, ManUA	Glc	+	
		Hot	Fuc, GulUA, ManUA			
3	*Chordaria magellanica*	Cold	Glc[a], Fuc, GulUA, ManUA		+	
		Hot	Glc, Fuc, GulUA, ManUA	Glc	+	0.7
	Punctuaria latifolia	Cold	Glc, Fuc, GulUA, ManUA	Glc	+	0.3
		Hot	Fuc, GulUA, ManUA			
5	*Desmarestia viridis*	Cold	Fuc, GulUA, ManUA			
		Hot	Glc, Fuc, GulUA, ManUA	Glc	+	0.15
6	*Coccofora langusta*	Cold	Glc[a], Fuc, GulUA, ManUA		+	
		Hot	Glc[a], Gal, Fuc, GulUA, ManUA		+	
7	*Pelvetia wrightii*	Cold	Glc[a], Fuc, GulUA, ManUA		+	
		Hot	Glc[a], Gal, Fuc, GulUA, ManUA		+	

[a]Glucose content (chromatographic evidence) in residues was small; laminarin was not isolated from these seaweeds. [b]With laminarinase from *S. sachalinensis*[6].

subsequent dialysis, the hydrolysates of the non-dialysable materials contained only glucose, and the susceptibility to the *endo*-laminarinase [β-(1→3)-glucan-glucanhydrolase] from *S. sachalinensis*[6] indicated the presence of β-(1→3) linkages. Data on these laminarins are given in Table II. For purposes of comparison, data are cited for the laminarin of known structure from *Laminaria hyperborea*.

TABLE II

DATA ON LAMINARIN STRUCTURE

Time collected	*Seaweed*	*Mol. wt.*	*M-chains* (%)	*G-chains* (%)	*Number of branch points per laminarin molecule*	
					Smith degradation	*Formic acid*
Aug. 1971	*Laminaria cycharioides* (cold)	5,000	89	11	2.4	2.5
Aug. 1971	*L. cycharioides* (hot)	4,900	86	14	1.6	1.5
June 1972	*L. cycharioides* (hot)	5,200	69	31		
June 1972	*L. japonica* (cold)	5,600	75	25	1.3	1.3
Aug. 1971	*Chordaria magellanica* (cold)	3,700	82	18		
Aug. 1971	*Ch. magellanica* (hot)	4,500	87	13	0.9	0.7
June 1972	*Ch. magellanica* (hot)	5,900	67	43		
Aug. 1972	*Punctuaria latifolia* (cold)	4,400	50	50	3.3	3.0
Aug. 1972	*Desmarestia viridis*	2,000	30	70		
	Laminaria hyperborea	3,000	56	45	0	0.1

Gel filtration on Bio-Gel P6 showed the laminarins to be of approximately the same molecular size. The molecular weights were determined by periodate oxidation and borohydride reduction[7,8]. The mannitol residue (M) attached to the laminarin

molecule (R) by a (1→6) linkage[3] yields 1 mol. of formaldehyde per polysaccharide molecule, whereas the reducing D-glucose end-group (G) yields 1 mol. of formaldehyde per molecule only after it has been reduced to D-glucitol[3,8]. The weak reducing-capacity of the laminarins indicates that mannitol is the terminal residue. Hence, by determining the yield of formaldehyde (μmole) from the laminarins before (n) and after reduction (n'), the M-chain [$(n/n') \times 100\%$] and G-chain contents, as well as the molecular weights, can be calculated.

The conditions for determining the molecular weights and M- and G-chain contents were chosen on the basis of kinetic curves (Figs. 1 and 2). Fig. 1 shows that the borohydride reduction is complete in two days, and that the formaldehyde liberated on subsequent periodate oxidation (Fig. 2) is maximal and should be determined[9] after 10 minutes. The G end-groups do not yield significant amounts of formaldehyde during this period; the model compound 3-*O*-methyl-D-glucose yields noticeable amounts of formaldehyde 3 h after the beginning of periodate oxidation. Sodium borohydride and sulphuric acid do not affect the absorbance, as shown by control experiments with solutions of glycerol (modified Nash technique[9]) and D-glucose (Nelson procedure[10]) before and after treatment with sodium borohydride solution pre-neutralised with sulphuric acid.

Table II shows that the laminarin molecular weights vary from 2,000 to 6,000; the highest molecular weight was observed for laminarins from seaweeds collected in June. On the other hand, laminarins from *L. cycharioides* and *Ch. magellanica*

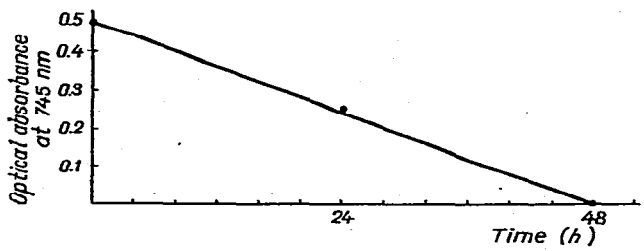

Fig. 1. Reduction of laminarin from *L. cycharioides* with sodium borohydride (Nelson's procedure).

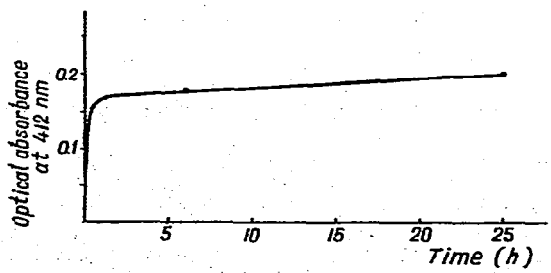

Fig. 2. Formaldehyde yield (Nash procedure) on periodate oxidation of laminarin from *L. cycharioides*.

collected in June have a lower M-chain content than those collected in August (possibly, the variation may be due to the collection of sample in different summers). The lowest M-chain content was observed in the laminarins from *P. latifolia* and *D. viridis*.

Paper chromatography of partial hydrolysates of each laminarin showed gentiobiose, in addition to laminaribiose and higher oligosaccharides, indicative of the presence of β-(1→6)-linked D-glucose residues. The proportion and location of such residues was established by means of Smith degradations (*cf.* ref. 3) which cleaved the laminarin molecule at each (1→6)-linked D-glucose residue.

Gel filtration on Sephadex G-25 of the Smith-degradation products (Fig. 3) revealed essentially a polysaccharide of high molecular weight and products of low molecular weight which yielded formaldehyde. These results indicate that the (1→6)-linked D-glucose residues are present solely as branches from the main β-(1→3)-D-glucan chain. The degradation products of the *L. hyperborea* laminarin are typical.

In contrast, Smith degradation of a lichenin [mixed β-(1→3)- and β-(1→4)-D-glucan[11]] gave a gel-filtration picture characteristic of an oligosaccharide mixture, and indicative of the presence of β-(1→4)-linked D-glucose residues within the main chain.

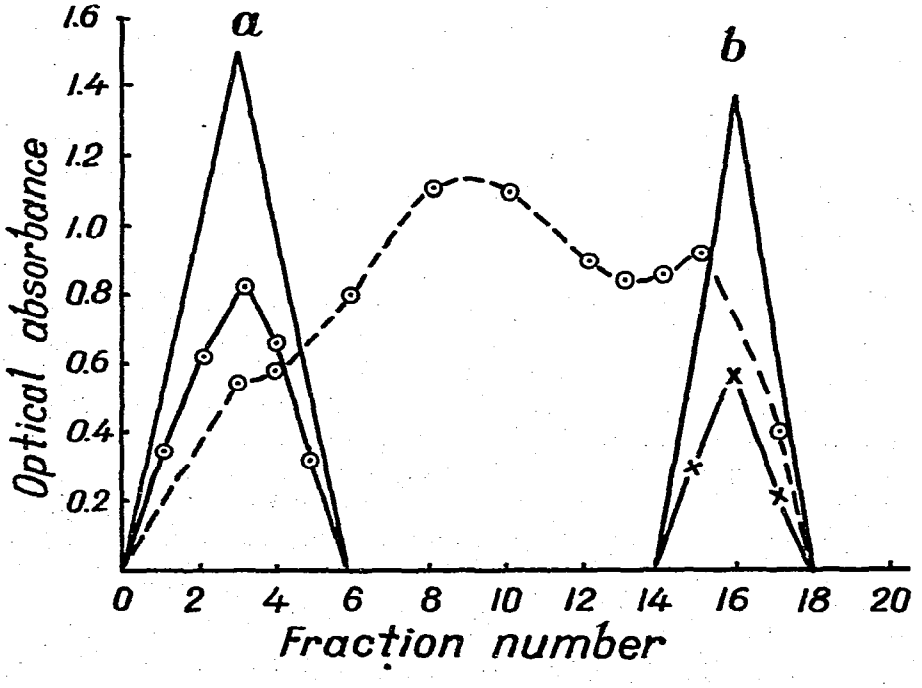

Fig. 3. Gel filtration on Sephadex G-25 of Smith-degradation products: ———, laminarin from *P. latifolia*; — — —, lichenin. Fraction analysis: ○, phenol–sulphuric acid method (at 480 nm); ×, modified Nash method after periodate oxidation (412 nm). Standards: a, starting laminarin; b, D-glucose.

From the Smith-degradation data, the average number of branch points per laminarin molecule may be determined on the basis of the molecular weights and the yield of formaldehyde from the reaction products of low molecular weight.

The time required (24 hours) for complete oxidation of the β-(1→6)-linked D-glucose residues was determined on the basis of the yield of formaldehyde following hydrolysis of the polyalcohol (Fig. 4). The β-(1→3)-D-glucan formed in the Smith degradation and isolated by gel filtration (Fig. 3) does not yield formaldehyde under these conditions. The yield of formaldehyde was determined by a modified Nash technique, and that of polyalcohol by the phenol–sulphuric acid method[12].

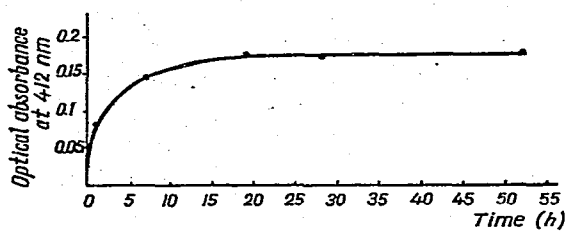

Fig. 4. Formaldehyde yield after Smith degradation of laminarin from *L. cycharioides*.

The number of branch points so determined (Table II) is in good agreement with the values obtained by titration of the formic acid released on periodate oxidation of the laminarins.

The laminarin from *P. latifolia*, isolated by cold extraction, has the largest number of branch points. A similar dependence on extraction procedure is seen by comparing the number of branch points in cold- and hot-extracted laminarins from *L. cycharoides*.

Methylation (Hakomori[13]) analysis of the laminarins confirmed the presence of (1→3) and (1→6) linkages. The products formed, which were identical to those obtained for the laminarin from *L. hyperborea*, corresponded to 2,4,6-tri-*O*-methylglucose, 2,3-di-*O*-methylglucose, and 1,2,4,6- and 2,3,4,6-tetra-*O*-methylglucoses (the last two compounds having the same mobility in g.l.c.). Peaks corresponding to 2,3,6-tri-*O*-methylglucose and 2,3-di-*O*-methylglucose were absent from the chromatograms, indicating the absence of (1→4) linkages in the laminarins. The proportion of 2,4-di-*O*-methylglucose indicated a substantial number of branch points.

Laminarins of defined structure are useful for studying the specificity of *exo*- and *endo*-laminarinases.

EXPERIMENTAL

General. — The following methods were used: phenol–sulphuric acid method[12] to determine polysaccharide, a modified Nash method for formaldehyde determination[9], and Nelson's method for determining reducing sugars[10].

Chromatography was performed on paper (FN-1) with 1-butanol–pyridine–water (6:4:3), and detection with aniline hydrogen phthalate.

The laminarin from *L. hyperborea* was obtained from Koch–Light Laboratories, Ltd.

A Tsvet-6 gas chromatograph (U.S.S.R.) with glass columns (3 mm × 1 m) containing 3% HFEFF-8-BP on Gas-Chrom 100–120 mesh was used at 125–225° (4°/min), the carrier gas being nitrogen (33 ml/min).

Isolation of laminarin. — Wet, ground seaweed, washed free of pigments with methanol, was extracted with 0.4% hydrochloric acid twice (at room temperature and at 50°) for 4 and 5 h, respectively.

The extracts were precipitated with methanol–1-butanol (3:1), with subsequent reprecipitation of the residues. An aqueous solution of the precipitate (collected by centrifugation) was treated with 0.5M Cetavlon until precipitation of acidic polysaccharide material was complete. The laminarin in the supernatant was precipitated with methanol–1-butanol, and an aqueous solution was dialysed against water and lyophilised.

Enzymic hydrolyses. — A solution of laminarinase (0.1 ml) was added to a 0.04% aqueous solution (0.4 ml) of polysaccharide, and the mixture was incubated at 37° for 10–40 min. The reducing capacity was then determined.

Acidic hydrolysis. — A weighed portion of polysaccharide was treated with (*a*) M H_2SO_4 at 100° for 2 h and (*b*) 62.5mM H_2SO_4 at 100° for 4 h.

The hydrolysates were neutralized with barium carbonate, deionised with Amberlite IR-120(H^+) resin, and analysed by p.c.

Reduction of laminarins. — Sodium borohydride (5 mg) was added to a solution of laminarin (10 mg) in water (1 ml). The mixture was kept in the dark at room temperature for 2 days, and then excess reductant was degraded by the addition of 50mM H_2SO_4 to pH 7.

Determination of formaldehyde. — A mixture (0.5 ml) of 0.02M periodic acid in 0.03M sodium hydrogen carbonate (pH 7) was added to a 0.1% aqueous solution (0.5 ml) of laminarin or laminaritol, and the mixture was kept in the dark at room temperature for 5–10 min. Excess periodic acid was degraded with 0.05M rhamnose (0.5 ml), and the Nash reagent[9] (1 ml) was added to the mixture. Absorbance was then measured at 412 nm with an SF-4A spectrophotometer. Mannitol was used as a standard.

Smith degradations[3]. — A mixture (10 ml) of 0.1M periodic acid in 0.15M sodium hydrogen carbonate (pH 7) was added to a 0.4% aqueous solution of laminarin (10 ml), and the mixture was kept in the dark at 4° for 24 h.

Excess periodic acid was degraded with M ethylene glycol (2 ml), and the polyaldehyde was dialysed against water till free from formaldehyde. The polyaldehyde was then reduced for 24 h with sodium borohydride (40 mg). The pH of the solution was adjusted to 7 and the polyalcohol was then hydrolysed with 50mM sulphuric acid for 48 h. The hydrolysate was neutralised (pH 7) with 0.1M sodium hydroxide.

Secondary periodate oxidation and determination of formaldehyde was then carried out by the above methods.

Each solution of laminarin-degradation products was evaporated to 1 ml and added to a column (1 × 40 cm) of Sephadex G-25. The column was eluted with dilute acetic acid, and fractions (1 ml) were analysed for sugars and formaldehyde (following periodate oxidation).

Determination of formic acid. — The formic acid formed on treatment of laminarin samples with 0.1M sodium metaperiodate in the dark at 4° during 24 h was titrated with 0.01M sodium hydroxide, using Phenol Red.

Methylation analysis. — Laminarin (100 mg), dried over phosphorus pentaoxide, was dissolved in dry methyl sulphoxide (5 ml) and methylated by the Hakomori method[13]. Methylation was repeated 2–3 times, until the product (~70 mg) had no i.r. absorption for hydroxyl groups.

The fully methylated polysaccharide was hydrolysed in a sealed ampoule for 3 h at 100° with methanol–72% hydrochloric acid (9:1). The mixture was neutralized with Dowex-1 x4 (HCO_3^-) resin, filtered, and evaporated *in vacuo*. The resulting mixture of methyl glycosides was analysed by g.l.c.

The products [2,4,6-tri-*O*-methylglucose, 2,4-di-*O*-methylglucose, and 1,2,4,6- and 2,3,4,6-tetra-*O*-methylglucoses (common peak)] from *L. hyperborea* laminarin were used as reference compounds, together with authentic samples of 2,3,6-tri-*O*-methylglucose and 2,3-di-*O*-methylglucose.

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