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SEPARATION AND CHARACTERIZATION OF THE SOLUBLE AND INSOLUBLE COMPONENTS OF INSOLUBLE LAMINARAN

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ABSTRACT

Insoluble laminaran, a (1→3)-β-D-glucan from *Laminaria hyperborea* (*L. cloustoni*), has been fractionated by differential solubility into soluble and insoluble fractions. These fractions were degraded with a purified exo-(1→3)-β-D-glucanase from *Basidiomycete sp.* QM806 giving, as primary hydrolysis products, D-glucose, gentiobiose, laminarabiose, and 1-*O*-β-laminarabiosylmannitol. Gentiobiose was obtained in only trace amounts from the insoluble fraction of laminaran, suggesting the absence of branching. Successive application of periodate oxidation, reduction, mild acid hydrolysis, and enzymic degradation indicated that the branch in the soluble fraction consists of a single β-(1→6)-linked D-glucosyl residue. The results indicate that "insoluble" laminaran is apparently an aggregate of three closely related polysaccharide species: a soluble, branched, reducing component (soluble laminarose); an insoluble, unbranched, reducing component (insoluble laminarose); and an unbranched, nonreducing component (laminaritol) that has a monosubstituted mannitol residue at the reducing terminal. Laminaritol was found to be about equally distributed between the soluble and insoluble fractions. The average d.p. of the laminaran components is 20–25 residues, as determined from the relative amounts of enzymic hydrolysis products and from periodate-oxidation data.

INTRODUCTION

Insoluble laminaran (laminarin)[1], a storage glucan in the fronds of the marine alga *Laminaria hyperborea* (*L. cloustoni*)[2], is a mixture of low-molecular-weight (1→3)-linked β-D-glucans which appear to be insoluble in cold water (20°) and which have variously been reported[3–6] to have a d.p. of 15 to 30.

Early work on insoluble laminaran indicated that it is heterogeneous, since approximately half of the chains are terminated by mannitol residues (laminaritol, or M-chains), whereas the other chains are reducing (laminarose, or G-chains)[7,8]. This

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heterogeneity was further substantiated when the reducing and nonreducing components of laminaran were separated by anion-exchange chromatography after oxidation of the reducing terminal to the corresponding acid[5].

Peat and coworkers[7] obtained 1-*O*-*β*-D-glucosyl-D-mannitol and 1-*O*-*β*-laminarabiosyl-D-mannitol by partial acid hydrolysis of insoluble laminaran, thereby revealing the nature of the linkage between the (1→3)-glucan chain and mannitol in the nonreducing component of laminaran. Isolation of gentiobiose and 3-*O*-*β*-gentiobiosylglucose from the same hydrolyzate provided the first evidence for (1→6)-linkages. Contrary to earlier results suggesting the presence of linear (1→6)-linkages[9–11], Manners and co-workers[3] were unable to detect 2,3,4-tri-*O*-methyl-D-glucose in methylated laminaran, and they concluded that the (1→6)-linkages represented branch points. This conclusion was supported by their periodate-oxidation studies. Thus, Smith degradation of laminaran eliminated glycerol from the nonreducing glucosyl terminal but did not effect internal fragmentation of the chain, which would have occurred if internal, linear, (1→6)-linked units had been present. Similar results had been obtained previously[12] when three successive degradations of laminaran by the Barry method gave a non-dialyzable product.

Comparison of the average chain-length ($\overline{c.l.}$) with the d.p. indicates that there are, on the average, 0.3 branch points per molecule of insoluble laminaran. Thus, it has been suggested that the primary difference between insoluble (*L. hyperborea*) and soluble laminaran (*L. digitata, L. saccharina*) is the degree of branching[3,13].

In a survey of laminaranases from a variety of sources, using insoluble laminaran as substrate, Chesters and Bull[14] tentatively identified 3-*O*-*β*-gentiobiosylglucose, 3,6-di-*O*-glucosylglucose, 1-*O*-*β*-glucosylmannitol, and mannitol-terminated laminara-oligosaccharides in their digests. These products are compatible with the other structural evidence, already cited and with the view that insoluble laminaran is a heterogeneous (1→3)-*β*-D-glucan having some chains terminated by mannitol residues and with occasional (1→6)-linked branches.

This communication clarifies the state of the heterogeneity of insoluble laminaran and the fine structure of the components of purified insoluble laminaran, as revealed by enzymic degradation with a purified exo-*β*-(1→3)-D-glucanase[15] (*β*-1,3-glucan glucanohydrolase, E.C. 3.2.1.39) from *Basidiomycete sp.* QM806. The exo-laminarase removes single glucosyl residues from the nonreducing terminal of substrates and bypasses one-unit, *β*-(1→6)-linked, branch-point glycosyl residues, releasing gentiobiose[16–18].

RESULTS AND DISCUSSION

The heterogeneity of insoluble laminaran[1] fractionation into soluble and insoluble components. — Commercial preparations of insoluble laminaran isolated from *L. hyperborea* contain impurities of low molecular weight. During dialysis of laminaran, the ratio of reducing capacity[19] to total carbohydrate[20] rapidly declined to a nearly constant value (initial, 11 mg/g reducing equivalent to glucose/total

carbohydrate; 9.1 in 10 h; 8.7 after 18 h), signifying an initial loss of readily dialyzable, low-molecular-weight, reducing material. The total carbohydrate continued to decline slowly, even after extended dialysis (initial, 1.028 g/ml total carbohydrate; 0.755 at 18 h; 0.612 after 48 h), indicating that laminaran is partially dialyzable because of its low molecular weight. A portion of the laminaran retrograded in the dialysis tubing after ~24 h at room temperature, producing a precipitate that showed streaming birefringence. Some of the laminaran, however, remained in solution, suggesting that a soluble species had been separated from an insoluble one.

Subsequently, commercial laminaran was separated into soluble* and insoluble fractions by repeatedly solubilizing laminaran in hot water and allowing it to precipitate spontaneously from solution during cooling. A soluble component of the laminaran was recovered from the supernatant solution by precipitation with acetone. Both the acetone-precipitated, soluble fraction and the insoluble fraction were refractionated by the same procedure, giving a series of soluble fractions and one insoluble fraction. The soluble fractions were similar in reducing capacity and in their behavior to the exo-laminaranase, as measured both by enzymic activity and chromatographic examination (paper), and were apparently similar in structure. Accordingly, they were combined for subsequent studies.

The purified, soluble-laminaran fraction is readily soluble in water at 20–25°, whereas the purified, insoluble laminaran dissolves only in hot water and precipitates as the solution cools. Apparently, in the original mixture, these two fractions, which occur in approximately equal portions, see Experimental and Table I, interacted and mutually aggregated, behaving essentially as an insoluble material. At low concentrations (0.1%) the purified, insoluble component is soluble at 60–70° and slowly retrogrades after several h at room temperature; at ~40° it remains soluble for extended periods of time.

No effort was made to recover all the laminaran quantitatively in the fractionation reported here. The purified fractions, whose structures are reported in this paper, account for 26% of the original crude, commercial laminaran. However, these two fractions appear to be representative of the total laminaran**†. Although the reducing-terminal heterogeneity of insoluble laminaran has been observed for some time[7,8,cf.21], this paper, to our knowledge, is the first report of a fractionation of unmodified laminaran into species having differing solubility.

Since production of laminaran by the seaweed shows seasonal variation[6], different samples of laminaran might show corresponding structural variations as well.

*This soluble fraction of *L. hyperborea* laminaran described is not to be confused with soluble laminaran isolated from *L. digitata* or *L. saccharina*, which are more highly branched and which are immediately cold-water-soluble upon extraction.

**An additional fraction, which was removed with the supernatant during the second cold-water wash, constituted the majority of the remaining original polysaccharide. It was subsequently separated into soluble and insoluble laminaran fractions, which respectively gave the same spectrum of exo-laminaranase hydrolysis products (see Experimental).

†In another study (N. Handa, unpublished), quantitative fractionation of laminaran freshly isolated from fronds of *L. hyperborea* also yielded approximately equal quantities of the soluble and insoluble fractions.

It is well known that the structure of laminaran varies with the algal species, and it has been suggested that commercially isolated laminaran may contain laminaran from mixed species[3] or artifacts produced during the extraction. To demonstrate that the two purified fractions studied here were not artifacts of the commercial extraction processes or due to seasonal variation or mixed algal species, laminaran was prepared directly from fronds of *L. hyperborea* collected at various seasons by using both commercial and more mild techniques. In addition, a typical sample of non-commercial crude *L. hyperborea* laminaran was obtained from Scotland. The various laminarans were fractionated by the procedure described in this paper and compared (before and after fractionation), by use of the exo-laminaranase, with commercial laminaran, commercial laminaran after dialysis (48 h), and the purified fractions already described. All samples showed essentially the same rate of hydrolytic action (±5%), the same reducing capacity, and the same pattern and ratio of oligosaccharides (paper chromatography) as the respective purified, commercial fractions.

On the basis of these results, the purified commercial laminaran studied here is representative of naturally occurring laminaran from *L. hyperborea*. Other studies[22] appear to corroborate this conclusion and there is no evidence thus far to suggest structural variations between different samples of laminaran from this species.

The nature of the (1→6)-linkage in laminaran. — Although there was no significant difference in the rate of hydrolysis of the soluble and insoluble fractions by the exo-laminarase, the hydrolysis products indicated a significant structural difference. Gentiobiose appeared only in the soluble-fraction digests.

The purified soluble and insoluble fractions were subsequently hydrolyzed enzymically on a preparative scale and the components were separated by charcoal-column chromatography. (A preliminary pilot hydrolysis indicated the optimal stage of enzymic action.) D-Glucose, laminarabiose, 1-*O*-D-glucopyranosyl-D-mannitol, 1-*O*-β-laminarabiosyl-D-mannitol, and laminaratriose, in addition to small amounts

TABLE I

EXO-LAMINARASE HYDROLYSIS PRODUCTS OF THE SOLUBLE AND INSOLUBLE FRACTIONS OF PURIFIED LAMINARAN (*L. hyperborea*)

Product	*Soluble laminaran*[a]		*Insoluble laminaran*[b]	
	(*wt, g*)	(*mmoles*)	(*wt, g*)	(*mmoles*)
Glucose	3.72	20.7	4.16	23.1
Gentiobiose	0.140	0.41	0.015	0.04
Laminarabiose	0.149	0.44	0.153	0.45
1-*O*-β-Laminarabiosylmannitol	0.234	0.46	0.288	0.57
1-*O*-β-Glucosylmannitol	0.019	0.05	0.011	0.03
Laminaratriose	0.025	—	0.004	—
Higher oligosaccharides	0.087	—	traces	—
Percent recovery	92		96	

[a]The total carbohydrate recovered was 4.37 g from 4.76 g of hydrolyzate. [b]The total carbohydrate recovered was 4.63 g from 4.81 g of hydrolyzate.

of other oligosaccharides, were obtained from both fractions, but gentiobiose was isolated in significant amount only from the soluble fraction (Table I). The trace quantities obtained from the insoluble fraction resulted presumably from incomplete removal of the soluble species. Since the exo-laminarase bypasses β-D-(1→6)-linkages in releasing gentiobiose[16,17], it is apparent that the soluble-laminaran fraction contains a β-D-(1→6)-linkage, probably as a branch point; whereas the insoluble-laminaran fraction contains no (1→6)-linkages and must consist of linear components. The solubility characteristics of the soluble fraction are consistent with its being branched.

The possibility of gentiobiose arising from a linear, intra-chain, (1→6)-linkage can be ruled out on the basis of the action pattern and specificity of the enzyme[17]. If such a linkage did occur at any place except at the nonreducing terminal, the enzyme would bypass the 6-substituted residue, cleaving the adjacent (1→3)-linkage on the reducing-terminal side[17]. In that case, instead of gentiobiose, a chain fragment would be released having a (1→6)-linked, reducing terminal, which would be degraded to 6-*O*-β-laminarabiosyl-D-glucose. Since the rate of enzymolysis of this trisaccharide resembles that of laminarabiose and is lower than that of 1-*O*-β-laminarabiosyl-D-mannitol[17], 6-*O*-β-laminarabiosyl-D-glucose would accumulate as a terminal product as do laminarabiose and laminarabiosyl-mannitol. This did not occur to a significant degree (if at all), as shown in Table I. The rapid release of gentiobiose from the soluble fraction during the course of the hydrolysis, as observed by paper chromatography, is thus not consistent with the presence of a linear, intra-chain, (1→6)-linkage. These results are in agreement with the methylation data of Annan *et al.*[22]

The length of the branch chain in laminaran. — The length of the branch in the soluble fraction of laminaran was determined by use of the Smith degradation, which utilizes in succession periodate oxidation, borohydride reduction, and mild acid hydrolysis[23]. The nonreducing terminals of the main and branch chains of (1→3)-linked glucans are removed, whereas the reducing terminal is converted into arabinitol. Repetition of the Smith degradation removes the newly generated, nonreducing-terminal residues and converts the arabinitol into a 2-substituted glycerol, which is immune to further periodate attack. (A 1-substituted mannitol terminal is converted into an ethylene glycol residue, which is also immune to further attack.) The process can be repeated any number of times, causing a sequential removal of the nonreducing terminals. Thus, the number of Smith degradations required for elimination of gentiobiose from the enzymic hydrolyzate indicates the number of residues in the branch.

The purified, soluble fraction of laminaran was subjected to the Smith degradation and, upon acidification, the oxidized and reduced laminaran precipitated from solution. The soluble fraction had thus been converted into an insoluble fraction by one Smith degradation, suggesting that debranching had occurred. This was confirmed when gentiobiose was not detected in the enzymic hydrolyzate. Thus, the branch in the soluble fraction of laminaran is only one residue long, as it was removed by one Smith degradation.

The enzyme, however, does not distinguish, in hydrolytic action, between a one-residue branch (see Fig. 1A) and a (1→6)-linked, nonreducing terminal (see Fig. 1C). It is known that the enzyme produces gentiobiose from a nonreducing-terminal (1→6)-linkage, since this structural arrangement was repeatedly encountered in the course of degradation of *Sclerotium* (1→3)-β-D-glucan[17,18]. These two types of linkage can, however, be distinguished by enzymolysis of laminaran that has been periodate-oxidized and reduced but *not* treated with dilute acid. Even though this derivative of laminaran has chemically altered, nonreducing terminals, it is still susceptible to enzymic attack, although at a much lower rate[16,17]. When the soluble fraction of laminaran that had been oxidized with periodate and reduced was subjected to hydrolysis by exo-laminarase, glycerol and a compound moving more slowly than glucose were detected by paper chromatography. The latter component was not observed in enzymic digests of the periodate-oxidized and reduced, insoluble fraction of laminaran. After isolation by paper chromatography, hydrolysis of the

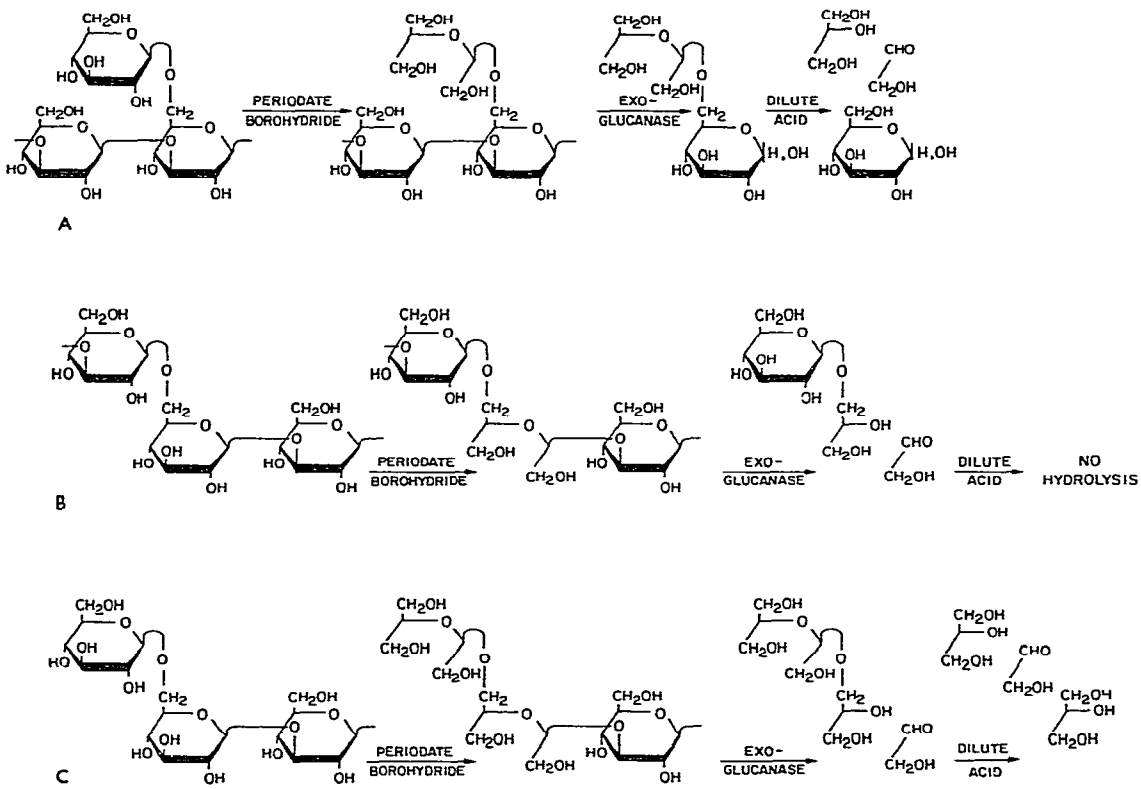

Fig. 1. Structural representation of periodate oxidation, borohydride reduction, exo-laminarase hydrolysis, and subsequent mild acid hydrolysis of a β-(1→6)-linkage Structures A–C represent the possible positions of the (1→6)-linkage in the soluble component of laminaran. Sequence A depicts a (1→6) branch; sequence B, a linear (1→6) intrachain linkage; and sequence C, a nonreducing terminal (1→6)-linkage.

unknown compound with 0.05M sulfuric acid for 18 h at 25° produced glucose and glycerol, which were detected by paper chromatography. This is the result expected from a single (1→6)-linked glycosyl branch, as illustrated in Fig. 1A. The enzyme releases what is essentially gentiobiose that has been selectively periodate-oxidized and reduced at the nonreducing end. Such a compound, during mild acid hydrolysis, would yield glucose, glycerol, and glycolaldehyde.

Linear (1→6)-linkages of the types shown in Fig. 1B and C would have been recognized by the appearance of either 1-*O*-β-D-glucopyranosyl-glycerol or the glycolaldehyde acetal of glycerol, respectively. These products are easily distinguished by their paper-chromatographic mobility and behavior toward acid hydrolysis. It appears, therefore, that the (1→6)-linkage occurs as a branch linkage in the soluble fraction, at some point other than at the terminals, and that the branch is a single glucosyl residue.

Other aspects of laminaran structure. — In the exo-laminaranase digests of the laminaran, the only mannitol-containing components were 1-*O*-β-laminarabiosyl-D-mannitol and its enzymic degradation product, 1-*O*-β-D-glucopyranosyl-D-mannitol, which appeared after extended incubation. Neither 1,2-di-*O*-β-laminarabiosyl-D-mannitol nor 1,2-di-*O*-β-D-glucopyranosyl-D-mannitol, which would have been predicted from previous studies[5], were detected. This is in agreement with results obtained using a purified *Rhizopus arrhizus* endo-laminarase[24] and various other partially purified laminarases[14]. The possibility that exo-laminarase selectively removes one of the laminarabiosyl chains from di-*O*-laminarabiosylmannitol can be discounted on the basis of its action pattern and the negligible rate of attack on 1-*O*-β-D-glucosyl-D-mannitol[17]. These results agree with the periodate-oxidation and the methylation data of Annan *et al.*[22], and it is thus evident that, in the nonreducing component of laminaran, the (1→3)-β-D-glucan chain is β-linked only to C-1 of mannitol.

Laminarabiose and laminarabiosylmannitol, arising from the reducing terminals of laminarose and laminaritol, respectively, occur in an approximately 1:1 molar ratio in the enzymic digests of both the soluble and insoluble laminaran fractions (Table I), indicating that about half of the chains are terminated by mannitol and the other half by glucose. The combined molar proportion of laminarabiose and laminarabiosyl-mannitol (Table I) indicates an average d.p. of ~25 for both the soluble and insoluble components of laminaran. This value is in accord with the periodate-uptake data presented in this paper and with the accepted literature value[4,6] of 20–25.

The presence of mannose in laminaran. — Mannose was not detected in the enzymic digests of the soluble and insoluble laminaran nor in acid hydrolyzates of the oligosaccharide fractions from the digests. However, small amounts of mannose were found in acid hydrolyzates of the material that remained at the origin during paper-chromatographic separation of enzymic digests of commercial laminaran. Thus, the mannose in the crude laminaran preparations is probably a constituent of a contaminating polymer and not an integral part of the laminaran. A similar result was

obtained by using a purified *R. arrhizus* endo-laminarase preparation[24], and other workers[4,22] have reached the same conclusion.

The number of molecular species. — From the foregoing discussion it is apparent that there are two molecular species of laminaran, laminarose and laminaritol, which vary in the nature of the reducing terminal; in addition, there are two molecular species, one linear and one branched, which are distinguished by solubility and by the generation of gentiobiose during enzymic hydrolysis. The present data indicate that the soluble fraction of laminaran contains both laminarose and laminaritol components, one or both of which is branched, since the enzymic digest contains gentiobiose. Likewise, the insoluble fraction also contains both laminarose and laminaritol components, which appear from enzymic analysis to be unbranched.

Although these results suggest the presence of four components: a branched laminarose and laminaritol in the soluble fraction, and linear laminarose and laminaritol in the insoluble fraction, the data (Table I) do not support this. The molar proportion of gentiobiose (0.41) in the soluble laminaran fraction is only half the combined proportion of laminarabiose, laminarabiosylmannitol (0.44+0.46), and glucosylmannitol (+0.05); therefore, only one of the soluble species could be branched. Since the only discernible distinction between the soluble and the insoluble fractions is the (1→6) linkage*, the unbranched, soluble species may be identical with one of the insoluble species. If this is the case, linear laminaritol may be the species common to both, as laminarabiosylmannitol is present in approximately equimolar amounts in both the soluble and insoluble fractions. The fact that nearly equimolar quantities of laminarabiose and gentiobiose were produced by degradation of the soluble fraction with exo-laminarase is consistent with this conclusion. Based on this assumption, the soluble laminaran fraction apparently contains approximately equal quantities of branched laminarose and a linear laminaritol, whereas the insoluble fraction consists of approximately equal quantities of linear laminarose and a linear laminaritol.

Thus, what has been termed *insoluble* laminaran[1] of *L. hyperborea* is an aggregate of at least three, structurally distinct, molecular species: an insoluble, linear, reducing component (linear laminarose), a linear nonreducing component terminated by a mono-substituted mannitol residue (laminaritol), and a soluble†, single-residue branched, reducing component (branched laminarose).

EXPERIMENTAL

Analytical procedures — Reducing capacity was determined by the Nelson–Somogyi method[19], total carbohydrate by the phenol–sulfuric acid method[20] and D-glucose by a modification of the D-glucose oxidase method[15,25]. Purified D-glucose oxidase and horse-radish peroxidase were obtained from Boehringer–Mannheim

*The techniques used cannot distinguish molecular-weight heterogeneity in any component, or sequence heterogeneity in the branched component. Such heterogeneity could give rise to additional components.

†See footnote, p. 65.

Corp., N.Y. Purified *o*-dianisidine was obtained from the Sigma Chemical Corp., St. Louis, Mo.

Descending analytical and preparative paper-chromatography were conducted with Whatman No. 1 and 3MM paper, respectively, with 1-propanol–ethyl acetate–water (7:1:2, solvent *A*; and 65:10:25, solvent *B*) and pyridine–ethyl acetate–water (2:5:7, solvent *C*). Compounds were visualized by a modification of the Trevelyan method[16,26].

Optical rotations were measured on a Bendix automatic polarimeter. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. All evaporations were carried out *in vacuo* in a rotary evaporator at 40–45° (bath temperature).

Enzyme preparation. — The exo-β-(1→3)-D-glucanase was a purified preparation obtained[15] from the culture medium of *Basidiomycete sp.* QM806; it was free from contaminating endo-laminarase, disaccharidase, β-glucosidase, and other β-glucanase activities as well as other known carbohydrase activities and did not catalyze transglucosylation reactions[17]. Although the enzyme at high concentrations will slowly degrade 1-*O*-β-D-laminarabiosylmannitol, gentiobiose, laminarabiose, and certain other disaccharides (see ref. 17), this action was negligible in this study, as confirmed by control experiments. There was also no action on 1-*O*-β-D-glucopyranosyl-D-mannitol[17]. The mode of attack, action pattern, specificity, and hydrolytic mechanism have been discussed elsewhere[16,17,24,27,28].

One unit of enzyme will produce[15,16] from laminaran 1 μmole of glucose perf min per ml of incubation mixture at 37° in acetate buffer, pH 4.8. Enzyme preparations were routinely checked (paper chromatography) for the presence o endogenous carbohydrate.

Fractionation of laminaran. — Commercial laminaran isolated from *L. hyperborea* (*L. cloustoni*) was obtained from the Liverpool Borax Co., St. Paul's Square, Liverpool 3, England. (The term *laminaran* used in the text refers to this preparation or purified fractions isolated from it, unless specified.)

Commercial laminaran (185 g) was stirred with water (750 ml) for 1 h at 25° and centrifuged. The colored supernatant solution was discarded and the residue was washed with water (400 ml) in the same way and the mixture was centrifuged. The supernatant, containing a loose gummy material, was decanted and the remaining hard-packed residue was washed twice with cold water, resulting in little change in the nature and volume of the residue. The residue was dissolved in hot water (1 liter, 60–70°) with addition of a few drops of ammonium hydroxide to ensure neutrality, kept at room temperature overnight and the insoluble laminaran that precipitated was collected by centrifugation. The supernatant solution was concentrated and treated with 5 volumes of acetone whereupon a soluble laminaran precipitated.

Solubilization of the cold-water-insoluble residue in hot water was repeated twice, giving finally a cold-water-insoluble laminaran fraction (16 g) and three cold-water-soluble laminaran fractions (total weight 28 g). The soluble laminaran fractions were each redissolved in hot water, centrifuged to remove any insoluble material, and

again precipitated with acetone. All fractions were washed successively with acetone and petroleum ether and dried *in vacuo* at 100°. The soluble fractions were indistinguishable and were subsequently combined. Both the insoluble and soluble fractions appear to be approximately equal in concentration in the original crude preparation (Table I).

Fronds of *L. hyperborea*, collected at various times during the year, were obtained from Dr. W. A. P. Black of the Institute of Seaweed Research, Inveresk, Scotland. Laminaran was extracted from the finely cut fronds by two different procedures: (*a*) extraction with 1.5% hydrochloric acid (10 parts by weight) for 12 h at 25°, and (*b*) extraction with hot water (55–65°) for 1 h. Each extract was decanted and the fronds were washed with water. The combined extract and washings were refrigerated overnight and the precipitated laminaran was collected by centrifugation and washed with cold water. By the procedure already described, the laminaran was separated into a soluble and insoluble fraction.

Periodate-debranched laminaran. — The soluble fraction (1% solution) was oxidized for 96 h in the dark at 4° with sodium metaperiodate (0.05M, pH 5). Periodate consumption, determined according to Dyer[29], was 0.265 moles per glucose unit at 72 h and increased slowly thereafter. Excess periodate and iodate were removed as the insoluble barium salts and the oxidized laminaran was reduced with a two-fold molar excess of sodium borohydride. After 12 h at 25°, additional borohydride (half of the initial quantity) was added and the solution was kept for 2 h, whereupon the pH was adjusted to 6.5 with acetic acid. A portion of this material was treated to remove borate as described previously[16] and retained for enzymic studies. Negligible hydrolysis of the laminaran derivative took place under these conditions or those used in the subsequent enzymic studies (see ref. 16).

The remaining solution was adjusted to pH 1.0 with 0.5M sulfuric acid, and kept for 18 h at 25°. (Upon acidification, the laminaran derivative precipitated as a white material showing streaming birefringence.) The insoluble material was centrifuged and washed with cold water until neutral and dried in a desiccator over phosphorus pentaoxide.

Enzymic hydrolysis. — Small-scale hydrolyses of the laminaran samples were conducted as follows: solutions (2 ml) of laminaran (0.5% in 0.05M acetate buffer, pH 4.80) were incubated at 37° with the exo-β-(1→3)-D-glucanase (0.1 unit) in test tubes stoppered with a glass sphere and stirred magnetically. Aliquots were removed at intervals, assayed for reducing capacity, and chromatographed (paper) to determine the extent of hydrolysis.

Large-scale hydrolyses of the soluble and insoluble laminaran fractions (5 g) were carried out under the same conditions, except that 50 units of enzyme were used. Aliquots were assayed periodically and examined by paper chromatography. Measured by reducing capacity, 78% hydrolysis of the soluble laminaran and 89% hydrolysis of the insoluble laminaran had occurred at the time (~14 h) when the hydrolysis was stopped.

The enzymic reaction was stopped by heating the hydrolyzates for 10 min in a

boiling-water bath. The hydrolyzates were cooled and passed through columns of a layered mixture of ion-exchange resins (Amberlite IR-120, H^+, in the top and bottom band, and Amberlite IR-45, OH^-, in the center band.) Equivalent amounts of the two resins were used in twice the amount required.

The deionized digests were concentrated to ~20 ml; no significant increase in acidity was noted (pH >3) and no new oligosaccharides formed by acid reversion were detected when aliquots were removed at intervals during the concentration step and examined by paper chromatography. The same conditions were employed in enzymic hydrolysis of the laminaran degraded by the Smith procedure (2 ml of incubation solution containing 0.5% substrate; 0.1 ml of enzyme at 37°). The incubation time was 24 h and the extent of hydrolysis 70–80%. The course of hydrolysis, as checked by paper chromatography, contrasted to that of the soluble component under identical conditions. This was also the case in the enzymic hydrolysis of the periodate-oxidized and reduced laminaran not subsequently subjected to dilute acid hydrolysis. The same paper-chromatographic analysis was also made.

Identification of the components of the enzymic hydrolyzates. — Aliquots (10 μl) of the deionized enzymic digest were examined by paper chromatography by using solvents *A* and *C* and authentic standards. Components of the large-scale hydrolyses were separated on a column of coconut charcoal (3.4 × 45 cm); D-glucose was eluted with 2.5% ethanol and the other components with stepwise increasing increments of ethanol (from 5–45% ethanol). Fractions were concentrated, chromatographed (paper, solvents *B* and *C*), and further purified when necessary by paper chromatography with solvent *B*. The oligosaccharides were dried to constant weight *in vacuo* and weighed (Table I).

The composition of each oligosaccharide was determined by hydrolysis with cation-exchange resin for 4 h at 95–100° followed by paper chromatography (solvent *C*). Aliquots were also removed at 30-min intervals to identify the products of partial acid hydrolysis. Each oligosaccharide was also characterized as follows for the enzymic products of the soluble laminaran fraction; the properties agree with accepted literature values.

Gentiobiose, $[\alpha]_D^{25}$ +7.15° (*c* 1.8, water), gave a crystalline β-octaacetate which was recrystallized from methanol, m.p. 194.5–196.5°, identical with authentic gentiobiose β-octaacetate.

1-*O*-β-D-*Glucopyranosyl*-D-*mannitol*, m.p. 140–141° and $[\alpha]_D^{24}$ −17.7° (*c* 1.0, water).

Laminarabiose, had $[\alpha]_D^{24}$ +18.6° (*c* 1.6, water) and gave a crystalline β-octaacetate, m.p. 160–161° and $[\alpha]_D^{26}$ −27.6° (*c* 1.0, chloroform).

1-*O*-β-*Laminarabiosyl*-D-*mannitol*, $[\alpha]_D^{26}$ −20.6° (*c* 4.4, water). It gave a crystalline acetate which was recrystallized from aqueous methanol, m.p. 146–147°. Application of the Smith degradation gave glycerol from the nonreducing terminal and 1-*O*-D-glucopyranosyl(ethylene glycol) from the other two residues; both were chromatographically identical with the authentic compounds.

Laminaratriose, $[\alpha]_D^{26}$ +1.8° (*c* 1.2, water); β-acetate, m.p. 121–122°.

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