The Enzymic Degradation of Laminarin

1. THE DISTRIBUTION OF LAMINARINASE AMONG MICRO-ORGANISMS

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(Received 2 April 1962)

β -Glucans occur in micro-organisms and higher plants as structural constituents of cell walls, as reserve materials and as extracellular substances of uncertain significance. Laminarin is such a reserve polysaccharide in many Phaeophyceae and it can be regarded as a mannitol-containing β -D-glucan in which the main polymeric linkage is β -(1 $\rightarrow$ 3). Enzymes hydrolysing β -D-(1 $\rightarrow$ 3)-glucans have been termed laminarinases by previous workers, since laminarin was used as the test substrate (Chesters, Apinis & Turner, 1956). Reese & Mandels (1959) suggested β -D-(1 $\rightarrow$ 3)-glucanase as a more general term for these enzymes. In the present study we have reserved the term laminarinase to describe the whole enzyme complex that participates in the degradation of laminarin and thus includes the β -D-(1 $\rightarrow$ 3)-glucanases. Data relating to the multiplicity of microbial laminarinases are discussed by Chesters & Bull (1963).

Since β -D-(1 $\rightarrow$ 3)-glucans occur in both fungi and higher plants, fungal β -D-(1 $\rightarrow$ 3)-glucanases are probably important in the intracellular mobilization of food reserves and in the extracellular hydrolysis of plant remains. Reserve polysaccharides, similar to laminarin in structure, have been isolated from various protozoans (see, for example, Archibald, Manners & Ryley, 1958), and Fellig (1960) has shown that a polysaccharide of this type in *Euglena* is made available to the organism by the action of a laminarinase.

EXPERIMENTAL

Materials. A single batch of insoluble laminarin (British Borax Co. Ltd., Liverpool) was used throughout the investigation. It was insoluble in cold water but passed into solution at 50–55°; precipitation occurred under static conditions at room temperature. The mannitol content, determined by periodate oxidation, was approx. 3%. Methylation analysis has failed to reveal the presence of D-mannose in this sample (Dr D. J. Manners, personal communication). Calcium and sodium alginates were supplied by Alginate Industries Ltd., Girvan, Ayrshire, Scotland. Celluloses were the products of Brown Co., Boston, Mass., U.S.A. (Solka Floc BW 40) and L. Light and

Co. Ltd., Colnbrook, Bucks. (soluble carboxymethyl-cellulose).

Micro-organisms. Bacteria, actinomycetes and fungi were obtained from the Department of Botany Culture Collection, University of Nottingham, and from other culture collections.

Culture conditions. Stock cultures were maintained on nutrient agar (bacteria and actinomycetes), potato dextrose-agar and/or laminarin-agar (majority of fungi), or on acidified malt-agar (basidiomycetes). In the preparation of laminarinase the organisms were grown at 25° in a liquid medium consisting of basal minerals (Taylor, 1951), insoluble laminarin (1.0%, w/v), peptone (0.25%, w/v), Difco yeast extract (0.25%, w/v), and Fe³⁺, Mn²⁺ and Co²⁺ ions (each at a concentration of 5 μ g./l.). A solid laminarin medium was obtained by the addition of 1.5% (w/v) of agar to these constituents. The final pH of the medium after autoclaving for 15 min. at 15 lb./in.² was 6.8–7.0; for basidiomycetes the medium was adjusted to pH 6.0. Marine fungi were grown on a Solka Floc-sea water medium and many other fungi gave better growth on a medium containing Solka Floc or starch. Marine yeasts were maintained on a medium comprising Difco yeast extract (0.3%, w/v), Oxoid malt extract (0.3%, w/v), glucose (1.0%, w/v) and sodium chloride (2.5%, w/v) in water (E. O. Morris & S. S. Ross, personal communication). All routine work was carried out on the shake-flask scale: 100 ml. narrow-necked conical flasks containing 40 ml. of inoculated medium were incubated on a reciprocal shaking machine (3 cm. throw at 120 cyc./min.). Larger batches of enzyme were prepared from cultures grown in 2 l. and 6 l. fermentation vessels. Inoculation was by suspensions of fungal spores or of bacteria in sterile water (approx. 10⁶ spores/ml.) or by small masses of mycelium for non-sporing species of fungi.

Enzyme preparation. Culture liquors were filtered through paper-pulp pads (30 cm. diam. $\times$ 2 cm. thick) and any remaining bacteria, spores or mycelial fragments were removed by passage through a sintered-glass filter (type 4 or 5). Filtrates were adjusted to pH 5.6–5.8 with 0.1 N-sodium hydroxide or 0.1 N-hydrochloric acid, then chilled to 2–3°. Enzyme material was precipitated from the filtrate with 2 vol. of acetone, at 2–3°. During the addition of acetone the filtrate was stirred continuously and precipitation was allowed to take place over 14–15 hr. The precipitate was centrifuged down at 1° (2000g for 20 min.), then dissolved in a minimum volume of glass-distilled water. A residual precipitate was removed by centrifuging. Enzyme solutions were dialysed in acetylated cellophan tubing (Dr D. R. Whitaker, personal communication) against glass-distilled water (five changes each of 5 l.). Finally, enzyme material was reprecipitated by the method

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described above, washed in several changes of cold ether and dried in a vacuum desiccator at room temperature. The resultant buff powder was stored at -12° .

Laminarinase assay. The activity of filtered culture liquors was demonstrated in penicillin-assay cups on opaque laminarin-agar for 24 hr. at 37° . Diameters of haloes produced as a result of laminarin hydrolysis were proportional to enzyme activities. Acetone-dried powders were assayed by incubating 2.5 ml. of laminarin solution (0.5%, w/v, in 0.05M-citrate-phosphate buffer, pH 5.8) with 2.5 ml. of a solution (0.1%, w/v) of acetone-dried powder in a buffer of the same composition and pH. After incubation for 30 min. at 37° , reducing sugars were determined (Somogyi, 1945). Laminarinase activity has been defined such that 1 unit of activity produces reducing sugar equivalent to 1 mg. of glucose under the conditions of incubation. Specific activities, i.e. units/mg. of protein, were obtained by using the Folin-Ciocalteu reagent for protein estimation (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

The majority of micro-organisms grew vigorously at 25° . Others with different temperature requirements were kept in incubators and hand-shaken at regular intervals. A laminarinase was produced by the large majority of bacteria, actinomycetes and fungi. In a survey of laminarinase production in fungi over 160 species from all major taxonomic groups were screened. More than 90% of all fungi investigated produced laminarinase, even when the carbon source for growth was cellulose or starch.

These fungi represented a wide range of ecological types and typical results are given in Table 1. A selection of marine ascomycetes and fungi imperfecti, kindly supplied by Dr S. P. Meyers, was assayed for laminarinase activity; only four species, *Halosphaeriopsis* sp. F115, *Culcitaina achraspora* F118, *Helicoma* sp. F151 and *Peritrichospora integra* F192, produced the enzyme and moreover activity was low. Marine yeasts studied, most of which were isolated from a trawl of fish (E. O. Morris, personal communication), were all laminarinase-positive but again activities were generally low. Even yeasts isolated from decaying *Laminaria* fronds, e.g. *Rhodotorula* sp. 12P, produced comparatively little enzyme. On the other hand many basidiomycetes possessed high activity against laminarin, in particular species of *Polyporus*, *Poria* and *Stereum*.

Cast seaweeds are readily attacked by many forms of animal life and by associations of other micro-organisms, but little decomposition occurs while the plants are immersed. To elucidate the role of each component in this complex community of micro-organisms is difficult but results of nearly a decade of seaweed research in this Department have shed some light on the problem. (i) Bacteria and actinomycetes were the most prolific colonizers of decaying weed and were isolated in the ratio of about 6:1. (ii) Actinomycetes were the most active laminarinase producers. (iii) Alginase pro-

Table 1. *Distribution of laminarinase among fungi*

Activity was measured by determining reducing sugars in the reaction mixture: 2.5 ml. of laminarin solution (0.5%, w/v, in 0.05M-citrate-phosphate buffer, pH 5.8) plus 2.5 ml. of 0.1% (w/v) solution of acetone-dried powder in buffer of the same composition and pH, after incubation at 37° for 30 min.

Source of fungus	Species	Activity (units/mg. of protein)
Land soil	<i>Cylindrocarpum radiculicola</i>	8.5
	<i>Penicillium violaceum</i>	30.3
	<i>Torula</i> sp.	19.4
	<i>Trichoderma viride</i>	340.0
Salt-marsh debris	<i>Cercospora salina</i>	34.0
	<i>Diplodina</i> sp.	3.1
	<i>Mortierella alpina</i>	6.2
Sea water	<i>Culcitaina achraspora</i> F118	3.2
	<i>Helicoma</i> sp. F151	5.6
	Marine yeast 13M (from cod gills)	19.6
	<i>Rhodotorula</i> sp. 12P	3.6
Decomposing textiles	<i>Aspergillus niger</i>	86.0
	<i>Chaetomium cochlioides</i>	42.0
	<i>Myrothecium verrucaria</i>	300.0
Rotting timber	<i>Polystictus versicolor</i>	72.0
	<i>Ustilina zonata</i>	28.5
	<i>Xylaria polymorpha</i>	79.0
Humic acid decomposers	<i>Penicillium luteum</i>	54.0
	<i>Trametes rubescens</i>	40.0
Keratin decomposers	<i>Epidermophyton floccosum</i>	25.7
	<i>Keratinomyces ajelloi</i>	14.7

Table 2. *Isolation of laminarin-decomposing micro-organisms from enrichment cultures*

Muds, or minced seaweeds, were mixed with sterile sand and exposed to a continuously percolating stream of laminarin solution (2.0%, w/v). Active organisms were isolated from the effluent and the mud- or seaweed-sand mixture on laminarin-agar.

Source of isolation	Percentage distribution			
	Actinomycetes	Bacteria	Yeasts	Other fungi
Seashore muds	10	67	0	23
Fresh minced seaweed	57	27	16	0

duction was more widespread among bacteria than among actinomycetes. (iv) Only 18% of the actinomycetes and 9% of the bacteria could utilize both laminarin and alginates. When seashore muds and minced seaweed fronds were used for the elective cultivation of laminarin-decomposers a pronounced difference in the composition of the microflora was apparent (see Table 2). The bacteria comprised Gram-positive, Gram-negative and Gram-variable forms but no group specificity was noticeable. Salt-marsh fungi seemed singularly ill-equipped to decompose cast weed, particularly lacking the ability to hydrolyse laminarin and alginates. We have found only *Cercospora salina* to have significant laminarinase activity and this species can also degrade sodium alginate, cotton cellulose and soluble carboxymethylcellulose.

DISCUSSION

The results of this survey, particularly when considered in conjunction with those obtained by Reese & Mandels (1959), strongly suggest that laminarinase is constitutive in fungi. Reese & Mandels (1959) found that a conidia-producing basidiomycete (QM 806), which was placed in the imperfect genus *Ptychogaster* Corda, was their best source of laminarinase, but that other basidiomycetes had low activity. However, we have observed high activities of this enzyme in many such fungi and it may be significant that the genus *Ptychogaster* is based on the imperfect states of the Polyporaceae, a family in which laminarinase activity is consistently high.

Microbial laminarinases were first reported by Chesters, Turner & Apinis (1955), who found that seaweeds decaying on the shore supported a wide range of micro-organisms. Streptomycetes provided the best source of laminarinase and *Corynebacterium pini* and *Bacillus polymyxa* were two of the most active bacteria isolated. Increasing amounts of sodium chloride limited the laminarinase and alginase activities of most isolated organisms, in contrast with the bacterial alginase prepared by Waksman & Allen (1934), which showed optimum activity in solutions containing added salt. In addition Chesters *et al.* (1955) found

that fungi occurring on cast weed hydrolysed laminarin only slowly. The current investigation agrees with these findings in as much as fungi isolated from salt-marsh debris had low activities: the one exception was *Cercospora salina*.

The physiology of laminarinase production in *Streptomyces* sp. S93 was studied in detail by Lester (1958). The enzyme was produced in deep culture and a method of selective precipitation evolved with ammonium sulphate. This semi-purified preparation was active against all β -glucosides tested, i.e. laminaribiose, cellobiose, gentiobiose, arbutin, amygdalin, salicin and aesculin, but had no action on cellulose. Laminarin was hydrolysed to glucose and a small quantity of 1-O- β -D-glucosyl-D-mannitol, but the latter was only slowly degraded. We have preferred to precipitate the enzyme with acetone since the use of ammonium sulphate and other conventional enzyme precipitants tended to inactivate the laminarinase.

Although considerable data are available on the degradation of individual constituents of seaweeds, little is known about the decomposition of seaweeds. McNaught, Smith & Black (1954) and Paton (1955) studied the breakdown of *Laminaria* fronds but no attempt was made to assess the role of the different components of the microflora concerned. Our own observations suggest that bacteria and actinomycetes are the most important organisms involved in this process. Indigenous marine fungi, particularly *Lulworthia* species, which are abundant in the ocean and flourish on submerged ligno-cellulose (Dr S. P. Meyers, personal communication), appear to be unable to attack seaweed products to any great extent. The only marine fungi to show significant laminarinase production have been species of *Rhodotorula* and other yeasts. This may be a consequence of β -D-(1 $\rightarrow$ 3)-glucan synthesis and mobilization in these organisms. Apart from *Cercospora salina*, which grows saprophytically on *Laminaria* and has morphological and reproductive characters in addition to its enzyme complement that point to a successful adaptation to the environment, it is remarkable that the power of fungi to degrade laminarin is almost entirely restricted to terrestrial species.

SUMMARY

1. More than 160 species of fungi and many other micro-organisms have been examined for their ability to produce laminarin-hydrolysing enzymes when grown in liquid culture.

2. Laminarinase is a constitutive enzyme in fungi.

3. Semi-pure laminarinase preparations have been obtained from culture filtrates by a method of acetone precipitation.

4. Methods of laminarinase detection and assay have been described and enzyme activity has been defined.

5. Enrichment culture techniques have been used to investigate the microflora concerned in the decomposition of seaweed. Bacteria and actinomycetes were found to be the most prolific colonizers of cast weeds, whereas fungi from a range of marine habitats had very low laminarinase activity and were relatively unimportant in the breakdown of cast weed.

Sincere thanks are offered to the following for their gifts of fungi: Dr S. P. Meyers (University of Miami); Dr E. O. Morris (Royal College of Science and Technology, Glasgow); Dr G. J. F. Pugh (University of Nottingham); Dr E. T.

Reese (Natick, Mass.) and Dr J. Savory (Forest Products Research Laboratory, Princes Risborough). A.T.B. is grateful to Boots Pure Drug Co. Ltd. for the provision of a research scholarship.

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Biochem. J. (1963) **86**, 31

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2. THE MULTICOMPONENT NATURE OF FUNGAL LAMINARINASES

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(Received 2 April 1962)

Accumulating information suggests that the enzymic degradation of polysaccharides conforms to stepwise cleavage by an exohydrolytic enzyme, to random fragmentation by an endohydrolytic enzyme, or to both patterns together. Thus β -glucan is hydrolysed by both exo- and endo- β -glucanases in barley, the endoenzyme also possessing the ability to hydrolyse laminarin and to produce a series of oligosaccharides (Preece, 1957). Several other polysaccharase systems are multi-component and recent studies on the degradation of chitin indicate that there exist an endochitinase which lowers the turbidity of chitin suspensions and

an exochitinase which produces *N*-acetylglucosamine (Berger & Reynolds, 1958; Lunt & Kent, 1960). 'Endohydrolysis' we define as that hydrolysis of laminarin which yields laminaridextrins as initial products; 'exohydrolysis' as that which yields glucose as the sole, initial product and only later yields laminaridextrins as a result of chain-shortening. The present work shows that the hydrolysis of laminarin by any fungus is brought about by a family of enzymes, the laminarinase complex, and strictly 'laminarinase' becomes an invalid term. However, we have retained the use of laminarinase to describe the whole enzyme complex (Chesters & Bull, 1963). This 'family of enzymes' concept was investigated by screening

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