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.

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The Enzymic Degradation of Laminarin

2. THE MULTICOMPONENT NATURE OF FUNGAL LAMINARINASES

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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 multicomponent 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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for exo- or endo-hydrolytic enzymes or both and by a more critical examination of selected systems by adsorption chromatography.

EXPERIMENTAL

Materials. Insoluble laminarin was supplied by the British Borax Co. Ltd., Liverpool. Amygdalin, cellobiose, gentiobiose and D-mannose were purchased from L. Light and Co. Ltd., Colnbrook, Bucks., and β-phenyl-D-glucoside from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Whatman diethylaminoethylcellulose, triethylaminoethylcellulose, carboxymethylcellulose and cellulose phosphate were purchased from H. Reeve Angel and Co. Ltd., London.

Preparation of laminaridextrins. Degradation of insoluble laminarin with fungal laminarinases yielded a series of oligosaccharides ranging from laminaribiose to laminarihexaose. These were adsorbed on charcoal (Norit; Flatters and Garnett Ltd., London) and displaced with ethanol–water mixture (Whistler & Durso, 1950). Glucose was removed by passing water through the column and the laminaridextrins then were eluted by a stepwise increase in the ethanol concentration.

Preparation of laminarinases. The methods of culturing micro-organisms, enzyme precpitation and assay were as described by Chesters & Bull (1963). Occasionally significant amounts of amylase were found in laminarinase preparations. Adsorption chromatography on carboxymethylcellulose (Whatman CM30), with a phosphate elution over the range 0·5–10 mM at pH 5·8, was adopted as a routine procedure to remove the amylase from such preparations.

Laminarin digests. Acetone-precipitated laminarinase (25 units), laminarin (0·5%, w/v) and citrate–phosphate buffer, pH 5·8 (5·0 ml.; 0·05M), were incubated together at 37° for 20 min. and the reaction was stopped by plunging the mixture into a water bath at 100° for 10 min. Reaction mixtures were analysed by ascending irrigation (20 hr.) on Whatman no. 1 paper in the solvent system propan-2-ol–acetic acid–water (67:10:23, by vol.) at 22°. Chromatograms were developed with alkaline silver nitrate (Burger, Hejmová & Kleinzeller, 1959) or the benzidine reagent of Horrocks (1949). R_{Glc} values have been defined as the ratio of the mobility of unknown sugars to the mobility of glucose. The identification of D-mannose in 24 hr. laminarin hydrolysates was confirmed by treating the hydrolysate with boiling methanolic hydrogen chloride (1%, v/v) for 6 hr. and separating the α-methyl D-mannoside so formed by paper chromatography with the solvent system pyridine–ethyl acetate–water (2:5:7, by vol.) (Professor F. Smith, personal communication).

Adsorption chromatography of laminarinases. (i) Calcium hydroxyapatite was prepared according to Tiselius, Hjerten & Levin (1956); preliminary washings with Nottingham tap water could be made without deleterious effects, thus economizing on the very large volumes of glass-distilled water normally required. Hydroxyapatite gels were stored in the dark at room temperature under 1 mM-phosphate buffer. Efficiency of enzyme separation was not influenced by the age of the gel. The most efficient column size was 1·5 cm.^{2} × 15 cm. and elution was carried out with phosphate buffer, increasing in concentration from 1 to 150 mM in a stepwise fashion. Applied pressure (3·5–6 lb./cm.^{2}) was necessary to attain the flow rate as stated by Tiselius *et al.* (1956). In some instances washed Solka Floc was used as a bulking agent. Purified wood cellulose had previously been used in this way (Steelman, 1958) without appreciably altering the column properties. (ii) Cellulose ion-exchange materials were prepared from the fine fraction of Solka Floc BW100 (Peterson & Sober, 1956), but later Whatman products became available. Several eluting systems, including phosphate and tris buffers, were investigated. Separations were performed at 1–2° and 2 or 5 ml. fractions collected on an automatic fraction collector (Towers and Co. Ltd., Widnes, Lancs.). Fractions were assayed against laminarin (Chesters & Bull, 1963) and protein was determined with the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951).

Paper chromatography of laminarinases. Acetone-precipitated enzyme in 0·05M-phosphate buffer was applied along a 6 cm. line, 5 cm. from the top of Whatman no. 4 paper. Irrigation was made at 1–2° by a descending flow of one of these solvent systems: (*a*) ammonium sulphate (30%, w/v), pH 6·8 (Simonart & Kwang Yü Chow, 1950); (*b*) acetone–water (1:1, v/v) (Jermyn, 1953); (*c*) sodium chloride (0·3M) in citrate buffer (0·05M), pH 5·6 (Gilligan & Reese, 1954).

Protein was detected with Solvay purple (Jones & Michael, 1950) or bromothymol blue (Papastamatis & Wilkinson, 1951). Enzyme movement was followed by spraying the chromatogram with laminarin solution (0·5%, w/v), incubating at 37° in a humidity chamber for 12 hr. and then spraying with a reagent for reducing sugars. Alternatively, the dried chromatogram was cut horizontally into 0·5 cm. strips and the enzyme assayed after elution.

RESULTS

Analysis of laminarin digests

Results of the paper chromatography of the products of enzyme hydrolysis of laminarin indicate three types of laminarinase action: (*a*) an intense glucose spot without (or with only trace amounts of) laminaridextrins, suggesting predominant exohydrolysis; (*b*) marked concentrations of laminaribiose and other laminaridextrins in the absence of (or in the presence of only trace amounts of) glucose, suggesting predominant endohydrolysis; (*c*) large concentrations of glucose,

Table 1. *Analysis of the laminarinase complex in* 160 *fungi*

Designation of exo- and endo-hydrolytic and intermediate types was based on the results of paper chromatography of laminarin–enzyme digests as described in the text.

Laminarinase type	Percentage of total fungi	Percentage of laminarinase positives
Exohydrolytic	20	25
Endohydrolytic	45	56
Intermediate	15	19
No significant production of enzyme	20	—

Table 2. *Variation in fungal laminarinases*

Enzyme components have been estimated from areas under the individual peaks and each is an average of two or more separations. Activity was measured by determining reducing sugars (as glucose) 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-precipitated laminarinase in buffer of the same composition and pH, after incubation at 37° for 30 min. Protein was estimated according to the scheme of Lowry *et al.* (1951).

Source of laminarinase	Enzyme components (%)		Units/mg. of protein (whole enzymes)
	Exohydrolytic	Endohydrolytic	
Marine yeast 13M	3	97	19
Rhizopus nodosus N245	17	83	71
Streptomyces sp. S93	20	80	24
Myrothecium verrucaria IMI25291	45	55	300
Basidiomycete sp. QM806	82	18	103
Trichoderma viride STR	92	8	340

laminaribiose and higher laminaribioses and higher laminaridextrins indicating approximately equal exo- and endo-hydrolytic activities (intermediate type).

The frequency of occurrence of these types is shown in Table 1. No correlation existed between the type of hydrolysis and fungal genera; indeed exohydrolytic, endohydrolytic and intermediate laminarinase types have been found in three strains of a single species, *Sporotrichum pruinosum*. Specific activities of many of these preparations have been reported (Chesters & Bull, 1963); no correlation was apparent between fungal habitat and type of enzyme activity.

The extent of laminarin degradation at intervals up to 24 hr. was followed by paper-chromatographic analysis. Five laminarinase preparations were selected to cover the whole range of laminarinase types from a predominantly exohydrolytic complex (*Trichoderma viride* STR) to a predominantly endohydrolytic complex (*Rhizopus nodosus* N245) (see Table 2). Mannitol-containing oligosaccharides were detected only in hydrolysates with laminarinase from *T. viride* and *Myrothecium verrucaria* IMI25291 (an intermediate type), and in both the rate of hydrolysis followed the decreasing order laminaritetraosylmannitol, laminaritriosylmannitol, laminaribiosylmannitol, β-glucosylmannitol. The two last-named compounds were extremely resistant to further attack and free mannitol was not detected. This resistance to hydrolysis agrees with the action of *Streptomyces* sp. S93 laminarinase on 1-*O*-β-glucosyl-D-mannitol (Lester, 1958). In the *M. verrucaria* digest glucose was present in appreciable quantities after 10 min. and thereafter gradually increased, although it never attained such high concentrations as in *T. viride* hydrolysates. Whereas laminaridextrins from the triose to the heptaose appeared rapidly and were steadily degraded, laminaribiose increased in concentration in all reaction mixtures and was only slowly hydrolysed. The mannitol-containing oligosaccharides were detected after about 100 min. but 1-*O*-β-glucosyl-D-mannitol was not produced until much later. 3-*O*-β-Gentiobiosyl-D-glucose slowly increased in concentration up to 90 min., after which time it was further degraded. Conversely, the gentiobiose concentration increased significantly after 90 min. and this may be due in part to trans-β-glucosylation, laminaribiose acting as the glucosyl donor. Similarly, cellobiose, which appeared in the 12 hr. sample, was probably a product of transglucosylation. A compound reacting with alkaline silver nitrate and having the same mobility as authentic 3,6-di-*O*-β-glucosyl-D-glucose (kindly supplied by Dr J. R. Turvey, University College of North Wales, Bangor) was observed in a few hydrolysates. In hydrolysates with *T. viride* and *M. verrucaria* laminarinases a sugar with R_{Glc} 1·11 was repeatedly detected and has been identified as D-mannose.

Fractionation of fungal laminarinases

Lester (1958) made a preliminary separation of the laminarinase complex from *Streptomyces* sp. S93 and eluted three components from a calcium hydroxyapatite column: one exohydrolytic (α) and two endohydrolytic (β and γ) enzymes. We have used this enzyme system in the development of a reproducible procedure for fractionating laminarinases and have also studied the effect of adding 1 part of Solka Floc to 1 part of hydroxyapatite. The presence of Solka Floc improved the flow rate and no pressure was necessary to attain a rate of 20 ml./hr. in a column 1·5 cm.^{2} × 15 cm., but it had an adverse effect on enzyme recovery, often 75–90% of the activity being lost. Various elution curves for the *Streptomyces* complex are illustrated in Fig. 1. Fractionation of laminarinases on cellulose ion-exchangers was usually accompanied by excessive spreading of the components, probably owing to heterogeneous binding of the protein on the exchanger. The use of Solka Floc as a bulking agent for hydroxyapatite columns was

abandoned and subsequently we fractionated all laminarinases on a cellulose ion-exchanger and refractionated individual components on hydroxyapatite. Such a procedure resulted in good enzyme recovery and the examples reproduced in Figs. 2–4 all represent recoveries of over 85 % of the original activity. Eight laminarinases were selected for fractionation after paper-chromatographic analysis of laminarin–enzyme digests had suggested the existence of a range of complex types.

Trichoderma viride *STR* (Fig. 2). Initial attempt at fractionation on hydroxyapatite resulted in the elution of a single exo-β-D-(1→3)-glucanase. A single component was also eluted from a diethylaminoethylcellulose column but residual enzyme eluted at higher molarities showed endohydrolytic activity. By using a bifunctional-exchange cellulose (cellulose phosphate) having weak and very strong acid groups it was possible to separate this small endo-β-D-(1→3)-glucanase, which amounted to about 8 % of the complex. When fractions were incubated with β-phenyl-D-glucose (1·0 %, w/v, in 0·5M-phosphate buffer) an additional component, *E*, was detected that could also hydrolyse laminaribiose, -triose and -tetraose, cellobiose and gentiobiose.

Basidiomycete sp. QM 806 (Fig. 3i). This species was used by Reese & Mandels (1959) in their work on laminarin hydrolysis and was cited by them as

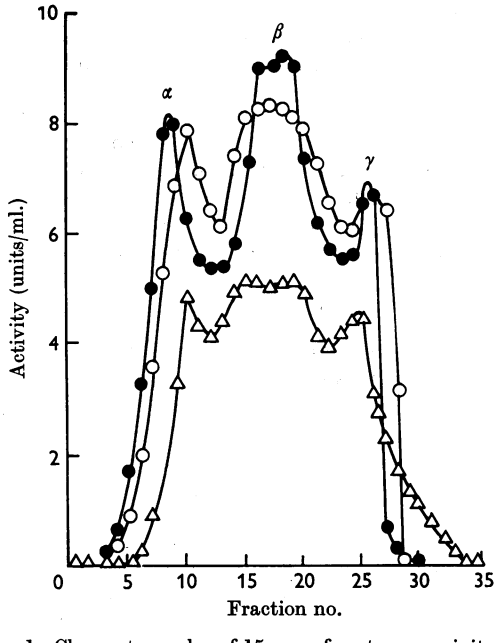

Fig. 1. Chromatography of 15 mg. of acetone-precipitated laminarinase, prepared from *Streptomyces* sp. S93, on a calcium hydroxyapatite column (1·5 cm. × 15 cm.) by stepwise elution with phosphate buffer, 1–150 mM, pH 5·8 (●); 1 part of Solka Floc BW40 to 1 part of hydroxyapatite was added as a bulking agent in some experiments (○). The elution diagram (△), obtained under similar conditions, is for laminarinase precipitated by ammonium sulphate (65 %, w/v) (adopted from Lester, 1958). Enzyme activities are expressed as mg. of reducing sugar (as glucose) liberated under the standard conditions of assay. α, Exo-β-D-(1→3)-glucanase; β and γ, endo-β-D-(1→3)-glucanases.

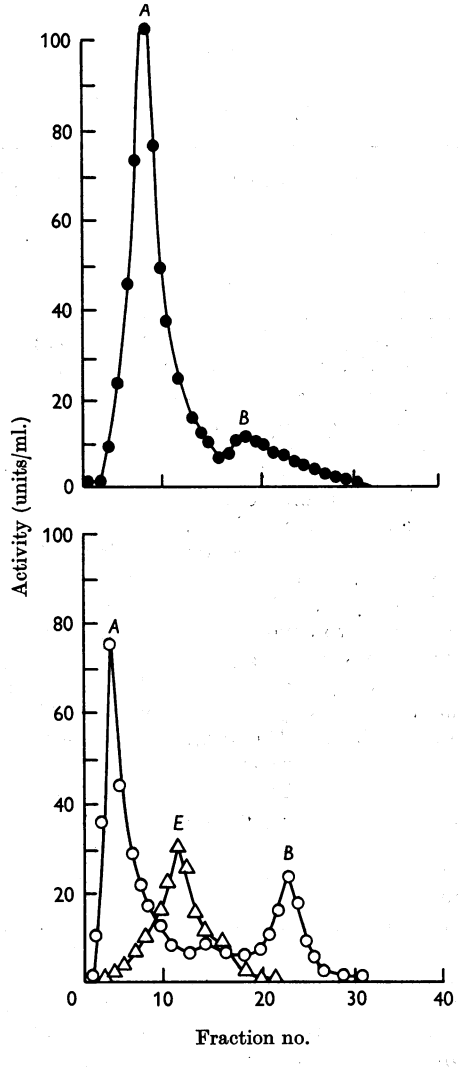

Fig. 2. Chromatography of 15 mg. of acetone-precipitated laminarinase prepared from *Trichoderma viride* STR on: ●, diethylaminoethylcellulose (1 cm. × 35 cm.) by stepwise elution with phosphate buffer, 1–150 mM, pH 5·8; ○, △, cellulose phosphate P70 column (1 cm. × 35 cm.) with the same elution conditions. Activity towards laminarin and β-phenyl D-glucoside were determined on each fraction as described in the text. *A*, Exo-, and *B* endo-β-D-(1→3)-glucanases; *E*, β-glucosidase.

a producer of exo-β-D-(1→3)-glucanase. Our laminarin-digest analysis indicated a major exohydrolytic component and this was well separated from a minor endohydrolytic component in diethylaminoethylcellulose.

Myrothecium verrucaria *IMI* 25291 (Fig. 3ii). Fractionation on hydroxyapatite resulted in the isolation of at least five components. An endo-β-D-(1→3)-glucanase, *B*, was displaced first from the column and had an activity of 0·925 unit/μg. of protein. Component *A*, 0·76 unit/μg. of protein, was exohydrolytic and unable to hydrolyse β-(1→6)-glucosidic linkages. Two smaller components, *C* and *D*, were endohydrolytic and, even after prolonged incubation with laminarin, glucose and short-chained laminaridextrins were not detectable. Two rather ill-defined peaks, *E* and *F*, had β-glucosidase activity of low specificity and were without action on laminarin. During enzyme fractionation often 20–25 % of the total activity was lost. Additional experiments were performed to investigate this phenomenon. A laminarinase solution that had a total activity of 100 units was fractionated on hydroxyapatite. When the eluted enzyme components were assayed individually only 78 % of the original activity was present, but when a proportionate mixture of the individual components was assayed, 94 % of this activity was recovered.

Marine yeast 13*M* (Fig. 3iii). Two large components were the main features of the laminarinase. Digests of laminarin and component *B* gave the typical pattern of sugars resulting from random fragmentation, whereas component *A* produced glucose rapidly and in large amounts. Gentiobiose was present in hydrolysates with component *B* and in fractions above tube 24. Very little β-D-(1→3)-glucanase activity was detected after these two large peaks had been eluted, though traces of exoglucanase were evident in fractions 32 and 40. Amygdalin (D-mandelonitrile β-D-gentiobioside) was used as a substrate for the assay of β-D-1→6-glucosidase activity in some experiments. This gentiobioside was hydrolysed in two different ways by the enzymes *E* and *F*. With *E* the gentiobioside–aglycone linkage was hydrolysed, with the production of gentiobiose; the action of *F* was to produce glucose in the absence of the disaccharide. The hydrolysis pattern with *F* corresponds to the action of almond emulsin, but we have seen no reports of amygdalin hydrolysis comparable with

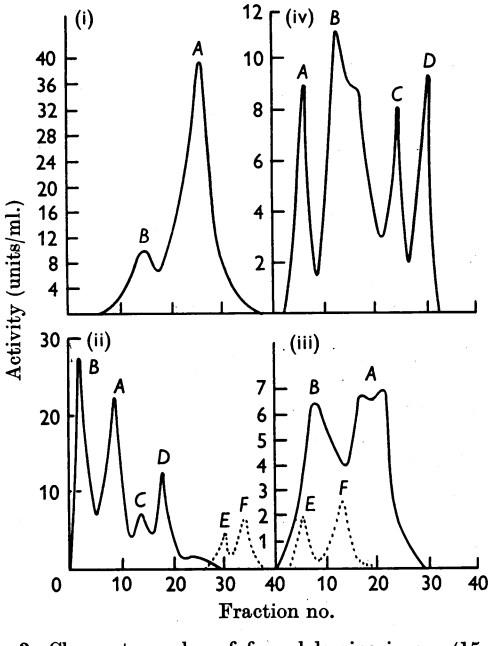

Fig. 3. Chromatography of fungal laminarinases (15 mg. of acetone-precipitated material) prepared from (i) basidiomycete sp. QM 806 on diethylaminoethylcellulose (1 cm. × 35 cm.), (ii) *Myrothecium verrucaria* IMI 25291 on hydroxyapatite (1·5 cm. × 15 cm.), (iii) marine yeast 13M on hydroxyapatite (1·5 cm. × 15 cm.), (iv) *Rhizopus nodosus* N 245 on cellulose phosphate (1 cm. × 35 cm.). Elution was by stepwise increase in phosphate buffer (1–150 mM, pH 5·8) and enzyme activities were determined on each fraction as described in the text. Experimental points are omitted for clarity. *A*, Exo-, and *B*, *C* and *D*, endo-β-D-(1→3)-glucanases; *E* and *F*, β-glucosidase.

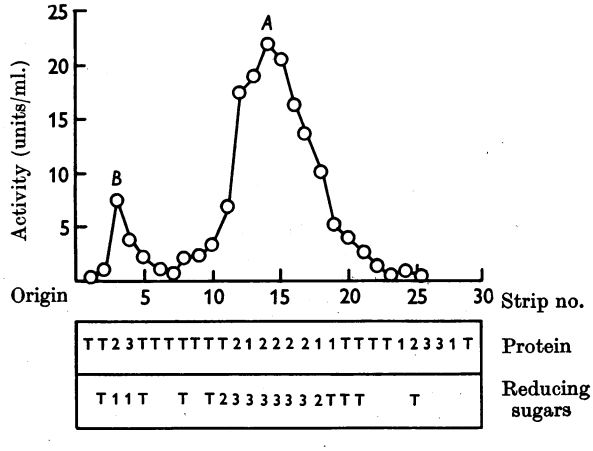

Fig. 4. Chromatography of 10 mg. of acetone-precipitated laminarinase prepared from *Trichoderma viride* STR on Whatman no. 4 chromatography paper. Irrigation was made at 1–2° by a descending flow of the solvent system sodium chloride (0·3 M) in citrate buffer (0·05 M). Activity towards laminarin, detection of protein and reducing sugars were determined and made on each fraction as described in the text. *A*, Exo-, and *B*, endo-β-D-(1→3)-glucanases. Concentrations: T, trace; 1, faint; 2, medium; 3, intense.

the action of component *E*. It is possible that both *E* and *F* act by producing gentiobiose, but that the *E* enzyme is unable to hydrolyse the β-D-(1→6)-linkage. The presence of gentiobiose is not considered to be due to glucose condensation since the disaccharide appears immediately in a glucose concentration of less than 0·3 % (w/v).

Rhizopus nodosus *N* 245 (Fig. 3iv). The use of cellulose phosphate (P70) gave good resolution of the laminarinase complex. Four β-D-(1→3)-glucanases were detected, one of which was exohyrolytic (*A*) and the remaining three were endohydrolytic (*B*, *C* and *D*). The endo-components did not appear to differ greatly one from another in action though the *B* enzyme brought about a more complete degradation of laminarin. It is likely that *C* and *D* resemble their counterparts in the *Myrothecium* complex: they are unable to hydrolyse laminaridextrins of a chain length less than about ten saccharide units. A β-glucosidase of low specificity was eluted in the same fractions as the exo-β-D-(1→3)-glucanase, but separation of these two activities was not achieved.

Cercospora salina. Two β-D-(1→3)-glucanases were resolved on hydroxyapatite. An endohydrolytic enzyme constituted two-thirds of the complex and was eluted first. The exohydrolytic component had a comparatively low activity, 0·4 unit/μg. of protein.

Penicillium stipitatum. In repeated experiments only one component was observed: an endohydrolytic enzyme. The preparation also possessed a low β-glucosidase activity. This was the only single-component laminarinase encountered and it may be noted that, in a study of the multiplicity of cellulolytic enzymes in fungi, only *Penicillium pusillum* had a single component (Gilligan & Reese, 1954).

Paper chromatography of fungal laminarinases

Results were not as clear as those obtained from column separations but confirmatory evidence was produced for several systems. The solvent used by Gilligan & Reese (1954) gave satisfactory separations of the *Streptomyces* sp. S 93, *Trichoderma viride* and *Myrothecium verrucaria* laminarinases. Solvay purple gave the clearest picture of protein movement. The small endo-β-D-(1→3)-glucanase observed in the *T. viride* complex was also detected by this method (see Fig. 4).

DISCUSSION

Stone (1957) found that the cellulolytic enzymes of *Aspergillus niger* could be separated from three laminarin-hydrolysing components by adsorption chromatography on calcium phosphate. This finding has been confirmed by chromatography on Dowex 1 columns (Krishna Murti & Stone, 1961), but some coincidence of laminarin- and cellobiose-hydrolysing activity was observed. These authors were unable to decide whether this coincidence was due to (*a*) the presence of linkage-specific β-glucosidases with similar physical properties, or (*b*) the presence of β-glucosidases whose specificities were not directed towards the position of the glucosidic linkage in the substrate. During the present investigations we have been able to separate β-glucosidase components of low specificity from the β-D-(1→3)-glucanases that are specific in action for laminarin. Our results are in close agreement with those of Duncan, Manners & Ross (1956), Reese & Mandels (1959) and Krishna Murti & Stone (1961), which suggest that specific β-(1→3)- and β-(1→4)-glucanases do exist. In addition we have been able to characterize the action of β-(1→3)-glucanases and have isolated both exo- and endo-hydrolytic components. We suggest that the hydrolysis of laminarin by fungi is by a family of enzymes, the laminarinase complex, which may comprise a β-glucosidase of low specificity, an exo-β-D-(1→3)-glucanase and one or more endo-β-D-(1→3)-glucanases. Table 2 indicates the variation in composition of fungal laminarinase complexes. The most active systems possess all three types of enzyme since the effect of the endo-degradation is to provide many more chains for attack by the exo-β-glucanase and β-glucosidase.

An overall activity loss of about 20 % was noticed during the fractionation of several laminarinases. This may have been due to (*a*) enzyme denaturation while adsorbed on the hydroxyapatite or cellulose, (*b*) insufficient elution, or (*c*) a synergistic effect between components.

We consider that (*a*) and (*b*) do not contribute seriously to this loss since a low working temperature was maintained and all protein material was eluted from the columns; further, a synergistic effect was clearly demonstrated in the *Myrothecium verrucaria* complex (see Fig. 3ii). The exo-β-D-(1→3)-glucanase (*A*) from this system can attack laminarin without preliminary cleavage of the molecule and the largest endo-enzyme (*B*) can continue random hydrolysis of short chains down to a chain length in the range six to ten saccharide units. The minor endo-enzymes (*C* and *D*) are specific for the larger dextrins and undegraded laminarin and consequently are more important during the early stages of hydrolysis. The action of the β-glucosidase (*E*–*F*) is significant in the light of possible limits to hydrolysis introduced by laminari-biose, -triose, -tetraose, -pentaose and β-(1→6)-glucosidic linkages.

The mechanism of exohydrolysis could follow three possible courses: (i) all molecules of laminarin are simultaneously degraded at a constant rate;

(ii) the first molecule is completely hydrolysed to glucose before the enzyme moves on to the next molecule; (iii) the enzyme acts in a randomized fashion with the formation of a range of molecular species.

Reese & Mandels (1959) favoured course (ii) because they were unable to detect the resistant dimer (laminaribiose) in their hydrolysates. They concluded that the absence of the dimer 'suggests the possibility of an "unzippering" action whereby each molecule of laminarin is completely hydrolysed to glucose before the enzyme moves onto the next molecule of substrate'. We have reached diametrically opposed conclusions. Laminaribiose has been found in substantial concentrations in exo-β-D-(1→3)-glucanase digests even when such enzymes were prepared from fungi used by Reese & Mandels (1959). Course (i) was discounted on the evidence of molecular-weight determinations on partly hydrolysed laminarin with periodate-oxidation analysis (Dr D. Walker, personal communication). Therefore, randomized action of the exo-enzyme is postulated. Reese & Mandels (1959) also found that when an exo-β-D-(1→3)-glucanase is added to an endo-β-D-(1→3)-glucanase digest of laminarin, instead of an overall increase in reaction occurring, the exo-enzyme was inhibited. These authors suggested that the short chains produced by the endo-enzyme were resistant to attack by the particular exo-enzyme preparation employed, basidiomycete sp. QM806 laminarinase. We investigated the possibility of final and complete hydrolysis of short-chain laminaridextrins being due to a non-specific β-glucosidase activity. The results of our experiments with β-glucosidases from marine yeast 13M, *Myrothecium verrucaria* IMI25291, *Rhizopus nodosus* N245 and *Trichoderma viride* STR indicated that such short-chain dextrins were completely degraded, the ease of hydrolysis following the sequence laminaritetraose > laminaritriose ≫ laminaribiose.

The present work suggests that exohydrolysis of laminarin liberates short-chain mannitol-containing oligosaccharides and, moreover, only when the exo-β-D-(1→3)-glucanase forms a major part of the laminarinase complex. If some mannitol is considered to be in a non-terminal position, then the results might be explicable on the grounds of a stereochemical hindrance, i.e. the endo-β-D-(1→3)-glucanase is unable to attack the molecule in the proximity of mannitol residues. In laminarinase systems where the exohydrolysis:endohydrolysis ratio is less than about 1·0 it is possible that the exo-enzyme preferentially attacks non-mannitol-terminated chains, and short-chain mannitol-containing oligosaccharides might then be expected to be released after a much longer incubation period. We have found this to be the case with laminarinases from *R. nodosus* N245 (four mannitol glucosides present after 96 hr.) and *Aspergillus niger* (strain from Dr Mikulus Burger, Czechoslovak Academy of Science, Prague) where laminaribiosyl- and β-glucosyl-mannitols were detected after 48 hr. Our observation of 3,6-di-*O*-β-glucosyl-D-glucose in some hydrolysates adds support for the hypothesis that there are some β-(1→6)-linked branch points in the laminarin molecule. Analysis of *T. viride* digests indicated a rapid production of D-mannose, the concentration remaining constant throughout the period of the experiments. D-Mannose was first observed in acid hydrolysates of laminaric acid by Smith & Unrau (1959); the present is the first report of its existence in laminarin–enzyme digests. However, this monosaccharide was detected in only a few of our experiments and it is questionable that it is a constituent of laminarin. Dr D. J. Manners (personal communication) has not detected D-mannose in our sample of laminarin with methylation analysis. Trans-β-glucosylation was very limited in the systems that we investigated. Significant synthesis of oligosaccharides such as cellobiose occurred only in digests containing a high proportion of exo-β-D-(1→3)-glucanase, undoubtedly a result of the high concentrations of laminaribiose, and possibly gentiobiose, that appear. A similar transient formation of oligosaccharides during the enzymic hydrolysis of cellobiose was reported by Crook & Stone (1957).

SUMMARY

1. The degradation of laminarin by fungi is achieved by a family of closely related enzymes. These enzymes were fractionated by adsorption and paper chromatography and have been found to comprise an exo-β-D-(1→3)-glucanase, one or more endo-β-D-(1→3)-glucanases and a β-glucosidase of low specificity. Individual enzymes have been obtained in a relatively pure state by refractionation on calcium hydroxyapatite and their action on insoluble laminarin and laminaridextrins was studied and compared with the action of the unfractionated complex.

2. Fungi almost invariably possessed both exo- and endo-β-D-(1→3)-gluconases; the sole exception was the laminarinase prepared from *Penicillium stipitatum*, which lacked an exohydrolytic component. A spectrum of laminarinase types has been recognized, ranging from those having predominantly exohydrolytic activity to those with a predominantly endohydrolytic action on laminarin.

3. An examination of the laminarinase of *Myrothecium verrucaria* IMI25291 suggested that the individual enzyme components exerted a synergistic effect on one another and a scheme of laminarin hydrolysis has been presented.

4. In a few laminarin hydrolysates D-mannose was repeatedly detected but chemical analysis of the laminarin used has failed to reveal its presence.

5. Mannitol and β-(1→6)-linked glucosides have also been observed during laminarin degradation and the tentative identification of 3,6-di-*O*-β-glucosyl-D-glucose in such hydrolysates suggested that β-(1→6)-linked branch points occur in the laminarin molecule.

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The Enzymic Degradation of Laminarin

3. SOME EFFECTS OF TEMPERATURE, pH AND VARIOUS CHEMICAL REAGENTS ON FUNGAL LAMINARINASES

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In previous papers we have reported on the distribution of laminarinase in micro-organisms and on its multicomponent nature (Chesters & Bull, 1963*a*, *b*). This paper discusses the effect of temperature, pH and certain chemicals on these enzymes. The methods of laminarinase preparation and fractionation should be mentioned here. These enzymes are very sensitive to many of the recommended enzyme precipitants, and ammonium sulphate, for example, in the concentration necessary for precipitation, severely inactivates the preparations. In this respect laminarinase differs from fungal cellulase, which can be fractionated with ammonium sulphate. Throughout this investigation acetone-precipitated enzyme preparations have been used. Analysis on the basis of amino acid composition cannot be used to distinguish between enzymes or even to differentiate enzymes and other proteins (Bailey, 1950), but a consideration of the active groups presented by the individual amino acids often sheds light on the nature of the activation. This has been demonstrated in part for laminarinase by a study of thiol groups.

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EXPERIMENTAL

Materials. Insoluble laminarin was supplied by the British Borax Co. Ltd., Liverpool. Laminaridextrins were given by Dr D. Walker and Dr W. J. Whelan, and were also prepared by us (Chesters & Bull, 1963*b*). Lactones