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 $\rightarrow$ 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 $\rightarrow$ 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 in-

vestigation 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.

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

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were purchased from L. Light and Co. Ltd. and glutathione from Hopkin and Williams Ltd. The lactone solutions were allowed to equilibrate for 5 days before use. All remaining reagents were British Drug Houses Ltd. AnalaR grade.

pH and temperature. A series of pH conditions over a range 3.2–7.9 was obtained by the use of Universal Buffer (British Drug Houses Ltd.). This mixed-salt buffer containing potassium hydrogen phthalate, phosphate, boric acid and sodium carbonate was employed to ensure ionic uniformity over the range of pH required. The pH values of the incubation mixtures were checked with an EEL direct-reading pH meter. Laminarinase activity was examined at the temperatures 10°, 20°, 27°, 30°, 37°, 42°, 45°, 55° and 60°.

Preparation of laminarinases. Methods of culturing fungi, enzyme precipitation and assay were as described by Chesters & Bull (1963*a*). In all experiments, acetone-precipitated laminarinase was used, from which amylase activity had been removed by adsorption chromatography as described by Chesters & Bull (1963*b*).

Laminarin digests. Laminarin (0.5%, w/v; 2.5 ml.) was incubated with 2.5 ml. of 0.1% (w/v) acetone-precipitated laminarinase solution for 30 min. at 37°. In experiments on the effect of temperature and pH on enzyme activity, incubation mixtures were prepared in the Universal Buffer at pH 5.8 or at the required pH value. Experiments to investigate the influence of various reagents on enzyme activity were performed in 0.05M-acetate buffer, pH 5.8 (Walpole, 1914).

Paper chromatography. Laminarin hydrolysates were analysed by descending irrigation on Whatman no. 1 paper in the solvent system butan-1-ol-pyridine-water-benzene (5:3:3:1, by vol.) of Green & Stone (1952) at 22°. Chromatograms were developed with alkaline silver nitrate (Burger, Hejmová & Kleinzeller, 1959).

RESULTS

Laminarinase activity and temperature

Initial studies were made with the laminarinase prepared from *Streptomyces* sp. S93, a complex comprising three β -D-(1 $\rightarrow$ 3)-glucanases, α , β and γ (Lester, 1958; Chesters & Bull, 1963*b*). With the unfractionated enzyme preparation activity was demonstrable over the whole temperature range

investigated, but with incubation periods longer than 30 min. the activity declined sharply at extreme temperature, particularly at 55° and 60°. When individual enzyme components were examined a significant displacement of the optimum temperature was evident between the exo- β -D-(1 $\rightarrow$ 3)-glucanase (α) and the endo-glucanases (β and γ), 27–30° and 37–42° being the respective optima. Similar experiments were carried out with laminarinases from other fungi. The latter were selected to contain examples of exohydrolytic, endohydrolytic and intermediate laminarinase types (Chesters & Bull, 1963*b*), and the results are summarized in Table 1. There is a distinct correlation between the complex type and the optimum temperature: the high value for *Penicillium stipitatum*, for example, can be correlated with an almost exclusively endo- β -D-(1 $\rightarrow$ 3)-glucanase activity. Only marine yeast 13M failed to fit into this general pattern: the predominantly endohydrolytic complex (97%) possessed a low optimum temperature of 30°.

A preliminary study has been made of the thermostability of these enzymes. Our results indicate that endo- β -D-(1 $\rightarrow$ 3)-glucanases are more thermostable than the corresponding exo-enzymes, a conclusion in close agreement with the heat-inactivation of cereal exo- β -glucanases and exo-xylanase (Preece & MacDougall, 1958). Other workers have reported a greater thermostability of exo- β -D-(1 $\rightarrow$ 3)-glucanases (Duncan, Mannes & Ross, 1956; Reese & Mandels, 1959) and a similar conflict on the thermostability of *Myrothecium verrucaria* cellulase is apparent in the results of Sanders, Sui & Genest (1948) and Kooiman, Roelofsen & Sweeris (1954).

Laminarinase activity and pH

In the following experiments incubation was carried out for 180 min. and enzyme activity determined after 30, 60 and 180 min. Blank titrations of the substrate solutions at each pH and without

Table 1. *Temperature optima of fungal laminarinases*

Activity was measured by determining reducing sugars (as glucose) in the reaction mixture, 2.5 ml. of laminarin solution (0.5%, w/v, in Universal 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 for 30 min. at the temperatures 10°, 20°, 27°, 30°, 37°, 42°, 45°, 55° and 60°. The type of laminarinase complex was determined by adsorption chromatography (Chesters & Bull, 1963*b*).

Preponderating activity of laminarinase	Fungus	Temperature optimum
Exohydrolytic	<i>Trichoderma viride</i>	30°
	Basidiomycete sp. QM 806	30
Intermediate	<i>Myrothecium verrucaria</i>	37–42
Endohydrolytic	<i>Rhizopus nodosus</i>	37–45
	<i>Penicillium stipitatum</i>	42–50
	Marine yeast 13M	30

Table 2. pH optima of β -D-(1 $\rightarrow$ 3)-glucanases from *Streptomyces* sp. S 93

Activity was measured over the pH range 3.2-7.9 at 37° according to the protocol in Table 1. β -D-(1 $\rightarrow$ 3)-Glucanases were separated on calcium hydroxyapatite as described by Chesters & Bull (1963b). Enzyme stability was determined by holding acetone-precipitated material in buffered solutions of known pH for 3 hr. at 1-2°, then measuring the activity under optimum conditions.

β -D-(1 $\rightarrow$ 3)-Glucanase	pH optimum			Stability
	30 min.	60 min.	180 min.	
Exo- (α)	5.2	5.2	5.2	Alkaline side of the optimum to pH 5.9
Endo- (β)	6.25	6.1	6.0	Acid side of the optimum to pH 5.6
Endo- (γ)	6.0	6.0	6.0	Both sides of the optimum to pH 5.8 and pH 6.25

enzyme were necessary to assess the true activity of the laminarinases, since the reducing power of the Universal Buffer varied slightly at different pH values. The β -D-(1 $\rightarrow$ 3)-glucanases from *Streptomyces* sp. S 93 were again used in initial studies and the results are given in Table 2. Subsequently the pH optima for several unfractionated laminarinase preparations were determined and these are given in Tables 3-5. Typical examples of unimodal, bimodal and intermediate pH-activity curves are illustrated in Fig. 1. The outstanding features of these results may be summarized as follows. (a) Laminarinases could be placed into three categories distinguished by one or two major activity peaks, or one major and one minor peak. (b) Several laminarinases were active over the whole pH range investigated, e.g. preparations from *Trichoderma viride*, *Penicillium violaceum* and *Myrothecium verrucaria*. (c) Other laminarinases were much more sensitive to pH changes and were inactivated at extreme values, e.g. preparations from *Aspergillus niger* and *A. oryzae*. (d) Laminarinase sensitivity to pH was not associated with either a predominantly exo- or endo-hydrolytic β -D-(1 $\rightarrow$ 3)-glucanase activity. (e) The form of the pH-activity curve showed some correlation with the type of laminarinase complex; bimodal curves were often related to intermediate complexes (*Myrothecium verrucaria*) and even curves having one definite optimum (*Trichoderma viride*) frequently showed a minor peak or shoulder that was probably the result of a small component (the endo-enzyme in *T. viride*).

Effect of metal ions and various chemical reagents

A wide range of reagents was selected, the choice of some being based on their use in previous studies (see Jermyn, 1952). Detailed investigations were made with two laminarinases, one exo- β -D-(1 $\rightarrow$ 3)-glucanase-dominant (*T. viride* STR) and the other endo- β -D-(1 $\rightarrow$ 3)-glucanase-dominant (*Rhizopus nodosus* N 245). In all the experiments the reagents to be tested were present in the assay medium at a concentration of 1 mM. Acetate buffer was preferred to citrate-phosphate since secondary effects

Table 3. pH-activity optima; unimodal curves

Activity of unfractionated, acetone-precipitated laminarinases towards laminarin was measured over the pH range 3.2-7.9 at 37° according to the protocol in Table 1.

Source of laminarinase	Preponderant activity of laminarinase	pH optimum
<i>Aspergillus niger</i>	Exo-	5.2-5.6
Basidiomycete sp. QM 806	Exo-	5.0
<i>Diplodina</i> sp.	Exo-	5.5-5.7
<i>Helicoma</i> sp.	Exo-	5.0
<i>Penicillium claviforme</i>	Intermediate	5.4
<i>Streptomyces</i> sp. S 93	Intermediate	5.4
<i>Trichoderma viride</i>	Exo-	4.5-4.7
Marine yeast 13 M	Endo-	5.5-5.8
Marine yeast 40 R	Exo-	5.0-5.3

Fig. 1. Laminarinase pH-activity curves: unimodal, *Aspergillus niger* (●); bimodal, *Cercospora salina* (○); intermediate, *Rhizopus nodosus* (△). Activity was determined over the pH range 3.2-7.9. A mixed salt buffer (Universal Buffer) was used to ensure ionic uniformity at each pH value. Reducing sugars (as glucose) were estimated in the reaction mixture, 2.5 ml. of laminarin solution (0.5%, w/v, in Universal Buffer of required pH) 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.

might have been induced by the chelating effect of certain reagents, resulting in the removal of phosphate ions. Results are expressed as percentage

activity or inhibition compared with control reagent-free digests and a 10 % variation has been taken as significant. Results from these experiments are given in Table 6. There was little difference between the two laminarinases with regard to particular reagent response, though the degree of response varied in several cases. This is well illustrated by the activation effect of Fe^{3+} , Mn^{2+} and Co^{2+} ions, and by the inhibition caused by β -indolylacetic acid, cyanide and phenylmercuri-

Table 4. pH-activity optima: bimodal curves
For experimental conditions see Table 3.

Source of laminarinase	Preponderant activity of laminarinase	pH optima	
		3.9-4.1	5.4-5.6
<i>Cercospora salina</i>	Endo-	4.0-4.5	5.5-5.6
<i>Irpex lacteus</i>	Intermediate	4.8-5.0	6.1-6.3
<i>Mortierella alpina</i>	Exo-	4.8-5.1	6.1
<i>Myrothecium verrucaria</i>	Intermediate	5.4-5.5	6.1-6.3
<i>Trichoderma album</i>	Exo-		

Table 5. pH-activity optima: intermediate curves
For experimental conditions see Table 3.

Source of laminarinase	Preponderant activity of laminarinase	pH optima	
		Major	Minor
<i>Aspergillus oryzae</i>	Endo-	5.3-5.6	6.0-6.1
<i>Penicillium violaceum</i>	Endo-	5.5-5.6	6.1
<i>Penicillium stipitatum</i>	Intermediate	4.7-5.0	5.8
<i>Rhizopus nodosus</i>	Endo-	4.5-4.8	6.3-6.4
<i>Sporotrichum pruinosum</i>	Exo-	4.4-4.6	6.1-6.2

Table 6. Effect of various reagents on laminarinase activity

Enzyme activity was determined in the presence and absence of the 21 reagents listed according to the experimental conditions described in the text. The effect of the reagents is expressed as an activation or inhibition when compared with control digests.

Reagent	Activation (%)		Inhibition (%)	
	<i>Trichoderma viride</i>	<i>Rhizopus nodosus</i>	<i>Trichoderma viride</i>	<i>Rhizopus nodosus</i>
Heavy-metal ions				
Fe ³⁺	24	13	—	—
Mn ²⁺	14	13	—	—
Co ²⁺	14	9	—	—
Zn ²⁺	6	6.1	—	—
Cu ²⁺	—	—	15	33.7
Ag ⁺	—	—	23	25.8
Hg ²⁺	—	—	100	87.5*
Metal-complex-forming agents				
KCN	—	—	78	22.7
Diphenylcarbazide	17.5	19	—	—
Nitroso-R salt	—	—	2.4	10.6
EDTA	—	—	2.8	3.5
Thiols				
Glutathione	17	3	—	—
Cysteine	9	11.4	—	—
Thioglycollic acid	—	—	4.9	6.6
Thiol reagent: phenylmercurinitrate	—	—	100	63*
Oxidizing agents				
Permanganate	24	19.4	—	—
Molybdate	14	18	—	—
Plant hormone: β -indolylacetic acid	—	0	20	0
Complex base: betaine hydrochloride	—	—	90.2	88.7
Lactones				
Galactono-(1→4)-	2.4	—	—	2.9
Glucono-(1→4)-	—	—	21	7.5

* 100% inhibition at 15mm concentration.

nitrate. Cyanide has been used for the detection of metal-ion catalysis but in fact is a very unspecific inhibitor and inactivates enzymes that are not metal-catalysed. In this respect the singularly mild effects of EDTA and Nitroso-R salt should be noted.

Phenylmercurinitrate. Laminarinase preparations gave a faint nitroprusside reaction (Arnold, 1911), indicating the presence of thiol groups in the preparations (cysteine and glutathione were used as controls). Phenylmercurinitrate at concentrations of 10 and 1 mM was added to digests, and control digests without the reagent were also incubated. The inhibition induced is recorded in Table 7. An increase in the proportion of exo- β -D-(1 $\rightarrow$ 3)-glucanase was accompanied by more extensive inhibition and paper-chromatographic analysis of digests supported this conclusion. Chromatograms of exo-enzyme action showed only very small traces of laminaridextrins, and glucose was hardly detectable. Enzyme preparations with a high ratio of endo- to exo-components still produced laminari-

dextrins in quantity but exohydrolysis was severely curtailed. In a second experiment glutathione was added to a similar reaction mixture and laminarinase activities were measured (Table 8). The protection given by reduced glutathione was considerable and laminarinase preparations with an endo-component exceeding about 20% were completely uninhibited by 1 mM-phenylmercurinitrate.

Glucono-(1 $\rightarrow$ 4)-lactone. The effect of this lactone on laminarin hydrolysis was studied at concentrations of 0, 1, 5, 10 and 100 mM. Blank titrations of the assay substrate without the enzyme were necessary to correct for the reducing power of the lactone. Six representative laminarinase complexes were investigated (Table 9). Paper chromatography of the hydrolysates indicated an accumulation of laminaridextrins with increasing lactone concentration, and the production of glucose fell sharply. Inhibition was accentuated by an increase in the exo- β -D-(1 $\rightarrow$ 3)-glucanase component (Fig. 2), but some degree of recovery was evident with all preparations (Fig. 3). Further experiments were per-

Table 7. *Inhibition of laminarinases by phenylmercurinitrate*

Enzyme activity was determined in the presence and absence of phenylmercurinitrate (1 and 10 mM) under the standard conditions of assay described in the text.

Source of laminarinase	...	...	...	<i>Trichoderma viride</i>	Basidiomycete sp. QM 806	<i>Myrothecium verrucaria</i>	<i>Rhizopus nodosus</i>
Concn. of phenylmercurinitrate (mM)	1	10		1	10	1	10
Inhibition (%)	100	100		100	100	70	77
						26	30

Table 8. *Effect of glutathione on the inhibition of laminarinase by phenylmercurinitrate*

Laminarinase activity was measured in a reaction mixture containing 2.5 ml. of laminarin solution (0.5%, w/v, in 0.05M-acetate buffer, pH 5.8), phenylmercurinitrate (1 mM), glutathione (1 mM) and 2.5 ml. of 0.1% (w/v) solution of acetone-precipitated enzyme in a buffer solution of the same composition and pH. Activity was measured under the standard conditions of assay.

Source of laminarinase	...	<i>Trichoderma viride</i>	Basidiomycete sp. QM 806	<i>Myrothecium verrucaria</i>	<i>Rhizopus nodosus</i>
Enzyme protection (%)		77	100	95	100
Residual inhibition (%)		23	0	5	0

Table 9. *Glucono-(1 $\rightarrow$ 4)-lactone inhibition of fungal laminarinases*

Enzyme activity was measured in the presence of lactone (100, 10, 5 and 1 mM) under the standard conditions of assay described in the text. Control digests without lactone were also analysed. Preponderant β -D-(1 $\rightarrow$ 3)-glucanase activity was determined by fractionation of laminarinases by adsorption chromatography (Chesters & Bull, 1963b).

Concn. of lactone (mM)	Inhibition (% of control digests)					
	92% Exo- $\leftarrow$ β -D-(1 $\rightarrow$ 3)-Glucanase activity $\rightarrow$ 97% Endo-					
	<i>Trichoderma viride</i>	Basidiomycete sp. QM 806	<i>Myrothecium verrucaria</i>	<i>Streptomyces S93</i>	<i>Rhizopus nodosus</i>	Marine yeast 13M
100	100	100	100	100	100	100
10	85	70	60	100	75	70
5	85	50	35	70	65	30
1	50	25	15	(2*)	30	(15*)

Enzyme activation.

Fig. 2. Laminarinase activities were determined in the presence of various concentrations of glucono-(1→4)-lactone. Activity was measured after incubation for 1, 3 and 5 hr. under the standard conditions of assay (Chesters & Bull, 1963*a*). Blank titrations on the substrate solution were performed to correct for the reducing power of the lactone at each concentration. ●, *Trichoderma viride*; ○, marine yeast 13M; ▲, *Myrothecium verrucaria*.

formed in which the laminarin was replaced in the reaction system by laminaridextrins (0.1%, w/v, of each of laminaribiose, laminaritriose, laminartetraose and laminaripentaose). The presence of glucono-(1→4)-lactone severely inhibited enzyme activity and paper-chromatographic analysis indicated that the order of inhibition was laminaribiose hydrolysis $\gg$ laminaritriose hydrolysis $>$ laminartetraose hydrolysis.

DISCUSSION

pH and temperature optima have been demonstrated for exo- and endo- β -D-(1→3)-glucanases; the latter have an optimum temperature approximately 10° higher than the exo-enzymes over a 30 min. incubation period and are the more thermostable of the two components. An incubation temperature of 37° has generally been used in our studies on fungal laminarinases. Fellig (1960) assayed laminarinase from *Euglena gracilis* at 37°, Crook & Stone (1957) used a temperature of 40° to study *Aspergillus niger*-laminaribiase activity, and Chesters, Apinis & Turner (1956) found that laminarin-hydrolysing enzymes from micro-organ-

Fig. 3. Laminarinase activity was determined in the presence of 100 mm- (○), 10 mm- (●), 5 mm- (Δ) and 1 mm- (▲) glucono-(1→4)-lactone. Activity was measured after incubation for 1, 3 and 5 hr. under the standard conditions of assay (Chesters & Bull, 1963*a*). Correction was made for the lactone-reducing power at each concentration. (a) *Trichoderma viride*; (b) marine yeast 13M; (c) basidiomycete sp. QM 806; (d) *Myrothecium verrucaria*.

isms were most active between 37° and 40°. Reese & Mandels (1959) reported maximum hydrolysis of laminarin at 60° over an assay period of 60 min. and that enzyme denaturation was rapid at 70°. The different results obtained by Reese & Mandels (1959) and ourselves are probably due to the different pH conditions employed. Whereas we have used pH 5.8, Reese & Mandels (1959) performed their assays under more acid conditions (pH 4.8) and it is significant that at pH 6.5 they found that enzyme denaturation occurred at much lower temperatures. Lester (1958) observed that, although laminarin hydrolysis was slow at 10° (*Streptomyces* sp. S93 system), degradation still continued after 5 days. This can be explained by laminarin precipitation, which occurs after about 10–12 hr. at 25° and earlier at lower temperatures. Thus temperature was partly an indirect factor in producing this result. Endo- β -D-(1→3)-glucanase

degradation of precipitated laminarin is restricted more than exohydrolysis: possibly this is a consequence of aggregation of molecular species leaving only small chains, or 'tails', which could be attacked by an exo- β -D-(1 $\rightarrow$ 3)-glucanase.

Apart from the *Streptomyces* sp. S93 studies, pH optima of individual laminarinase components were determined for several fungi including *Cercospora salina*, *Myrothecium verrucaria* and *Trichoderma viride*. The exo- and endo- β -D-(1 $\rightarrow$ 3)-glucanases could be distinguished by their different pH optima, and the various peaks in the pH-activity curves probably represent distinct components. Jermyn (1959) investigated the effect of pH on fungal β -glucosidases and pointed out that interpretations of the variations in pH-activity curves in terms of multicomponent enzymes would have been unprofitable since crude enzymes were used. The present work was conducted with preparations of much greater purity and with separated enzymes and we consider such variations to be significant. The results from 20 fungal laminarinases placed the pH optima of exo- and endo-enzymes at 4.9–5.0 and 6.0–6.1 respectively.

The pH optima of β -D-(1 $\rightarrow$ 3)-glucanases from oats, barley, potato, hyacinth, soya bean, actinomycetes and seaweeds are close to our values (Dillon & O'Colla, 1951; Peat, Thomas & Whelan, 1952; Duncan *et al.* 1956; Preece, Garg & Hoggan, 1960). Polysaccharases from other marine organisms require more alkaline conditions for optimum activity: agarase, pH 6.0 (Yaphe, 1957); α -L-(1 $\rightarrow$ 2)-fucanase, pH 7.0 (Yaphe & Morgan, 1959); carrageeninase, pH 7.5 (Yaphe & Baxter, 1955); alginase, pH 8.0 (McDougall, 1958).

Both exo- and endo-enzymes were activated by Fe^{3+} , Mn^{2+} and Co^{2+} ions, but extensive dialysis suggests that they are not specifically ion-activated. The effect is probably a process similar to the metal catalysis of non-enzymic hydrolysis. The ion is thought to be loosely bound to both the enzyme and substrate, thereby inducing configurational or energetic changes which catalyse the reaction. Fellig (1960) found that Mn^{2+} ions, at the same concentration as used in our experiments, activated the *Euglena* enzyme by 50–100%; Fe^{3+} ion was a mild inhibitor and Hg^{2+} ion produced a marked inhibition. But Mn^{2+} and Co^{2+} ions activated cellulases from *Poria vaillantii* (Sison, Schubert & Nord, 1958) and Basu & Whitaker (1953) found Zn^{2+} ion to have a stimulating effect on *Myrothecium verrucaria* cellulase. Both exo- and endo- β -D-(1 $\rightarrow$ 3)-glucanases were activated by oxidizing agents, probably by their action on reduced thiol groups, and this can be compared with the stimulation of carboxymethylcellulase by permanganate and ferrieyanide (Jermyn, 1952).

In this study we were interested to discover

what type of substance produced enzyme inactivation. The inhibitory effect of heavy-metal ions gave no discrimination between cellulase and carboxymethylcellulase (Toyama, 1956), but exo- and endo- β -D-(1 $\rightarrow$ 3)-glucanases showed a different degree of response to certain reagents. The effect of Cu^{2+} , Hg^{2+} and Ag^{+} ions and of phenylmercurinitrate was indicative of inactivation via thiol groups. The latter are among those which effect the formation of enzyme-substrate complexes, and according to Barron (1951) three types of thiol group occur in proteins: freely reacting, sluggish (giving a negative nitroprusside test but forming mercaptides) and masked (not exposed until the protein configuration is altered). At least some free thiol groups occur in laminarinase preparations but it cannot be stated whether these or other undetected thiol groups are the responsible active centres. We can conclude, however, that Hg^{2+} , Cu^{2+} and Ag^{+} ions and mercurinitrate act by forming reversible mercaptides with certain thiol groups. The results show that the exo-enzymes are preferentially inhibited (Table 7); β -amylase is also inactivated preferentially by thiol reagents (Englard, Sorof & Singer, 1951). When glutathione is present in the system, competition for mercaptide formation occurs and the phenylmercurinitrate reacts with the tripeptide and not with the enzyme.

The criterion of gluconate or galactonate inhibition has been used to separate β -glucosidases into two groups, the 'taka' and 'emulsin' types. However, these distinctions are not absolute. Jermyn (1959) found that all the β -glucosidases he examined were of the 'taka' type and similarly we have found fungal β -D-(1 $\rightarrow$ 3)-glucanases to be gluconolactone-inhibited. The extent of inhibition can be related to the size of the exo-component: the *Trichoderma viride* complex, of which over 90 % is exo- β -D-(1 $\rightarrow$ 3)-glucanase, is inactivated almost completely by all but the 1 mM-lactone treatment. Recovery of activity was most complete in endo- β -D-(1 $\rightarrow$ 3)-glucanase-dominant laminarinases, e.g. marine yeast 13M and *Myrothecium verrucaria*, and is greatly enhanced by the presence of even a small endohydrolytic component (cf. the basidiomycete sp. QM 806 complex, which has 15 % random activity). A consideration of hydrolysis rates of short-chain laminaridextrins sheds some light on the mode of glucono-(1 $\rightarrow$ 4)-lactone inhibition. We have shown previously that there is no differential hydrolysis of laminaridextrins except that laminaribiose is hydrolysed at a significantly slower rate (Chesters & Bull, 1963b). Glucono-(1 $\rightarrow$ 4)-lactone severely inhibits hydrolysis of these short-chain residues which accumulate and cause a blockage in the overall degradation. The β -glucosidases of low specificity thus appear to be inhibited, a conclusion agreeing with Jermyn's (1959) results. Short-

chained cellodextrins are not hydrolysed in a preferential order (Grassman, Zechmeister, Toth & Sadler, 1933; Youatt, 1958), and inhibition of cello-dextrin hydrolysis decreases as the chain length increases from two to five saccharide units (Festenstein, 1959). Festenstein showed cellobiase activity to be 99 % inactivated by 2.5 mM-glucono-(1→4)-lactone, and we suggest that inactivation of these simple β -glucosidases is very critical in the total hydrolysis of both cellulose and laminarin.

Two points should be emphasized in the light of our results on microbial laminarinase systems: the possible physical properties of laminarinase components from different sources and the degree of purity of the preparations. Krishna Murti & Stone (1961) have discussed physical aspects of fractionated β -glucosidases and suggest that the following are important in evaluating their action: (a) different forms of proteins may possess a similar biological activity; (b) changes in protein molecules due to hydrolysis of peptide or amide bonds may occur without loss of biological activity; (c) part of a protein complex may be associated with other material and this may be responsible for the appearance of several components.

Thus the conflict in thermostability encountered between our results and those of Reese & Mandels (1959) and Duncan *et al.* (1956) may be an expression of the physical properties of laminarinases from different sources and of different levels of purity. Whitaker (1960), commenting on the thermostability of *Myrothecium verrucaria* cellulases, suggests that such reports are not incompatible since many enzyme-substrate complexes are much more stable than the individual free enzymes. Since our enzyme preparations did not represent single protein species it is possible that the effect of certain reagents, e.g. the activating effect of ions, could be indirect. In addition, the positive nitroprusside test may have been given by some contaminating protein: however, the experiments with thiol reagents are highly suggestive of thiol groups being implicated in the active centres of laminarinases.

SUMMARY

1. Various effects of pH, temperature and chemical reagents on fungal laminarinases have been recorded and an attempt has been made to correlate these data with the multicomponent nature of these enzyme systems.

2. The exo- and endo- β -D-(1→3)-glucanases investigated showed different temperature optima under the conditions of assay. Generally endo-hydrolytic enzymes appeared to be more thermostable than exo-enzymes, though certain endo-hydrolytic enzymes had a low temperature optimum. Exo- β -D-(1→3)-glucanases exhibited opti-

mum activity at pH 4.9–5.0, whereas endo-enzymes were most active at pH 6.0.

3. Fungal β -D-(1→3)-glucanases were not specifically ion-activated enzymes, though several metal ions enhanced activity; the decreasing order of catalytic efficiency was Fe^{3+} , Mn^{2+} , Co^{2+} , Zn^{2+} ions.

4. Exo- β -D-(1→3)-glucanases were preferentially inhibited by thiol reagents but could be protected or reactivated by reduced glutathione. Laminarin hydrolysis was also curtailed by the action of glucono-(1→4)-lactone; this was chiefly a result of specific inactivation of β -glucosidase components.

5. The results have been discussed in the light of enzyme purity and the influence of contaminating protein, and from the viewpoint of variations in physical properties of laminarinases from different sources. Such factors may explain conflicting results which appear in published reports and in this paper.

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Biochem. J. (1963) **86**, 46

The Activities of some Particulate and Non-Particulate Oxidative Enzymes in Carrot Tissue

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As part of a study of respiratory pathways in carrot tissue, some aspects of malate metabolism by slices and mitochondria prepared from carrot tissue have already been reported (see Dalgarno & Birt, 1962*a*, *b*). In amplification of this work the enzymic constitution of the extramitochondrial fraction of carrot tissue has been investigated. No comprehensive study of the preparation and properties of a soluble fraction from a similar tissue was available, although James & Richens (1960*a*) have described such a fraction from carrot tissue. However, only one enzyme was assayed (pyruvate decarboxylase) and no general comparisons with soluble fractions prepared by other methods are possible.

This paper describes various procedures for the preparation of soluble fractions from carrot tissue, and the assay of a number of oxidative enzymes in these fractions. The possibility that the soluble malate dehydrogenase is capable of generating reduced nicotinamide nucleotides for oxidation by the mitochondria is discussed in relation to further data on the interaction between extramitochondrial enzymes and the mitochondrial oxidase system.

MATERIALS AND METHODS

Reagents. Apart from those specified below, the organic acids, including DL-isocitrate (trisodium salt), amino acids, coenzymes and enzymes, used were supplied by Sigma Chemical Co., St Louis 18, Mo., U.S.A., or were from the sources described by Dalgarno & Birt (1962*a*, *b*). Spermine was obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.; glutathione was obtained from S.A.F. Hoffmann-La Roche Co. Ltd., Basle, Switzerland;

the alcohol dehydrogenase used was prepared according to the method of Racker (1950); the cytochrome *c* was a gift from Dr R. Henderson.

Preparation of soluble fractions from carrot tissue

Soluble fractions prepared from tissue disrupted in a blender. The homogenizing procedure used for the preparation of a mitochondrial fraction (see Dalgarno & Birt, 1962*a*) was suitable as a first step in the preparation of a soluble fraction from carrot tissue. All operations were carried out at 0–4° in a cold-room. In experiments with a medium of sucrose (0.44 M) in phosphate buffer (sodium and potassium salts, 0.1 M, pH 7.1) containing 1% (w/v) bovine serum albumin, the homogenate was squeezed through two layers of muslin and the filtrate centrifuged at 0° in a Spinco model L ultracentrifuge at 70 000g for 90 min.; the supernatant liquid was removed, saturated with ammonium sulphate (200 g.) and recentrifuged at 70 000g for 45 min. The considerable precipitate obtained after this centrifuging was resuspended and homogenized in approximately 15 ml. of phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1). In preparations with spermine (20 mM), or without albumin in the homogenizing medium, the procedure was identical except that the ammonium sulphate precipitate (in smaller amount) was most effectively removed by resuspension and filtration of the contents of the centrifuge tubes through Whatman no. 1 paper in a Buchner funnel. The sediment was scraped from the paper and homogenized in approx. 8 ml. of phosphate buffer. Fractions prepared without albumin were dialysed (with stirring) against phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1) for at least 2 hr. before use; continuation of dialysis overnight did not alter the activity of malate dehydrogenase in this fraction. Fractions prepared with albumin were always dialysed overnight. The soluble fractions obtained from the ammonium sulphate precipitates were used immediately, or after a period of storage at –15° (the duration of storage is noted in the