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SOME ASPECTS OF THE SPECIFICITY AND ACTION PATTERN OF β-1,3-GLUCAN GLUCANOHYDROLASE FROM *SPISULA SACHALINENSIS*

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SUMMARY

1. The study of some aspects of the specificity and action pattern of laminarinases (β-1,3glucan glucanohydrolase, EC 3.2.1.6) from *Spisula sachalinensis*, which we had previously referred to as laminarinases III and IV (V. V. SOVA *et al.*, *Biochim. Biophys. Acta*, 212 (1970) 111) has been carried out. Both enzymes can probably be ascribed to endo-β-(1→3)-glucan glucanohydrolases.

2. Some data on homogeneous laminarinase IV have been reported.

INTRODUCTION

In a previous communication[1], we reported on the purification and some properties of homogeneous β-1,3-glucan glucanohydrolase (EC 3.2.1.6) from the marine bivalvia *Spisula sachalinensis*. Certain interesting properties of this laminarinase (high specific activity, mol. wt. 22 000, and thermostability) led us to its further study. The present paper explains some aspects of the specificity and the apparent action pattern of both homogeneous and inhomogeneous laminarinases IV and III, respectively, as well as some of their characteristic features.

MATERIALS AND METHODS

The determination of laminarinase activity has been described earlier[1]. Glucose was determined by a glucose oxidase method according to the procedure with reagents TCM-III obtained from Biochemica Test Combination (Germany). The reducing sugars were determined in accord with NELSON[2]; control over the carbohydrate concentration was effected by the phenol–H_2SO_4 method[3].

Substrates

Soluble laminarin from *Laminaria cycharioides*[1] was used. Insoluble linear laminarin from *Laminaria hyperborea* and certain laminaridextrines were kindly

supplied by Dr. A. Bochkov (N. D. Zelinsky Institute of Organic Chemistry, Acad. Sci. U.S.S.R., Moscow). Air-oxidized and reduced laminarins were prepared in accord with another known method[4], while laminarin subjected to periodate oxidation and reduced with $NaBH_4$ was prepared in accord with the method in ref. 5. Xylan was originally obtained from the seaweed *Nemalion vermiculare* by Dr. Usov of the same Institute, and kindly supplied by him. Oat and barley glucans were kindly supplied by Prof. S. Kirkwood (Department of Biochemistry, Minnesota University, U.S.A.) Other polysaccharides were commercial reagents.

Modification of enzyme purification method

A method of enzyme purification, previously suggested by SOVA *et al.*[1], was somewhat altered, since lyophylization and subsequent dialysis of the product led to a loss in activity. In order to avoid this procedure stage, gel filtration on Biogel P-30 was carried out directly in a 0.05 M succinate buffer (pH 5.2). The combined active fractions of the P-30 column were placed (without lyophylization and dialysis) on a SE-Sephadex column in the same buffer. As a result, we avoided loss in activity and obtained a somewhat greater product yield.

Other procedures

When necessary, enzyme transfer into the acetic buffer was carried out by gel filtration of enzymatic fractions on Biogel P-2 in a 0.05 M acetate buffer (pH 5.6).

Amino acid composition was determined on an automatic amino acid analyser, type 6020 A (Czechoslovakia). NMR spectra were recorded using a "Varian" spectrometer, 100 MHz, in deuterium oxide at 45°.

RESULTS

Some data on laminarinase IV

Amino acid analysis

Table I shows the amino acid composition of laminarinase IV.

Effect of metal ions, possible inhibitors and certain detergents

In order to investigate the effect of these substances on the activity of laminarinase IV, 0.1 ml of the enzyme (0.05 unit) were mixed with 0.4 ml of 0.05 M acetate buffer (pH 5.6), containing a corresponding metal ion or potential inhibitor and detergents, and heated for 15 min at 37° (in some cases, the time was increased). Then, 2.0 mg of the substrate in 0.5 ml of the same buffer were added, and laminarinase activity was measured using our regular method for determining reducing sugars. The results are cited in Table II.

Kinetics

Values K_m and v_{max} for laminarinase IV were determined with laminarin from *L. hyperborea* (mol. wt. 4320, 24 glucose residues) and laminarin from *L. cycharioides* (mol. wt. 5600). To determine K_m, the known LINEWEAVER AND BURK[6] method was used. Laminarin hydrolysis occurred in the range in which the reaction rate was limited by substrate concentration (from 0.4 to 0.04 mg/ml). For results see Table III.

TABLE I

AMINO ACID COMPOSITION OF LAMINARINASE IV

The hydrolysis was performed with 6 M HCl at 110°, 24 h. Cysteic acid and methionine were determined by a separate analysis using the method of ref. 18.

Amino acid	*% Total protein*	*Residues per molecule (mol. wt. 22 000)*
Alanine	4.67	12
Arginine	5.48	7
Asparatic acid	12.29	20
Cysteic acid	8.68*	11
Glutamic acid	9.37	14
Glycine	5.96	17
Histidine	5.84	8
Isoleucine	3.89	6
Leucine	4.62	8
Lysine	4.63	7
Methionine	4.87	8
Phenylalanine	4.38	6
Proline	4.75	9
Serine	6.84	14
Threonine	6.39	12
Valine	7.30	9

Effect of laminarinase IV on laminaridextrines

0.5 ml of the solution, containing 0.5 mg of the corresponding substrate, were mixed with 0.5 ml (0.05 unit) of the enzymatic solution in a 0.1 M acetate buffer (pH 5.6). The activity of laminarinase IV was determined by the glucose oxidase method, and the results are cited in Table IV.

TABLE II

EFFECT OF METAL IONS AND SOME REAGENTS ON THE ACTIVITY OF LAMINARINASE IV

Metal ions, reagents	*Final conc. (mM)*	*Relative activity*
Control	—	(100)
Pb^{2+}	$1 \cdot 10^{-3}$	65
Cu^{2+}	$1 \cdot 10^{-3}$	30
Ag^{+}	$1 \cdot 10^{-4}$	18
	$1 \cdot 10^{-5}$	55
Hg^{2+}	$1 \cdot 10^{-4}$	0
	$1 \cdot 10^{-5}$	45
Monoiodoacetic acid	$1 \cdot 10^{-3}$	95
p-Chloromercuribenzoate	$1 \cdot 10^{-4}$	1
	$1 \cdot 10^{-5}$	85
Sodium azide	$1 \cdot 10^{-3}$	100
Ethylenediamine tetra-acetic acid	$1 \cdot 10^{-3}$	95
	$1 \cdot 10^{-2}$ (24 h)	3
Sodium lauryl sulphate	$1 \cdot 10^{-3}$	2
Sodium diethyldithio-carbamate	$1 \cdot 10^{-3}$	90

TABLE III

THE MICHAELIS CONSTANTS (K_m) AND MAXIMUM REACTION VELOCITIES (v_{max}) FOR THE HYDROLYSIS OF LAMINARINS WITH LAMINARINASE IV

Substrate	*Relative enzyme units*	K_m *(M)*	v_{max}, *(moles/ml per min)*
Laminarin from *L. hyperborea*	(1)	$3.08 \cdot 10^{-5}$	0.625
Laminarin from *L. cycharioides*	2.5	$2.86 \cdot 10^{-5}$	1.62

TABLE IV

RELATIVE VELOCITY OF HYDROLYSIS OF SOME LAMINARIDEXTRINES WITH LAMINARINASE IV

Substrate	*Relative velocity*
Laminarin from *L. hyperborea*	(1000)
Laminaribiose	35
Laminaritetraose	394
Laminaripentaose	580

Certain aspects of enzyme specificity and action pattern

Effect of laminarinases III and IV on laminarin

The effect of the enzymes we have purified was observed and compared with that of exo-laminarinase from *Basidiomycete QM 806*[4,7,8], endo-α-(1→3)-glucanase from *Trichoderma viride*[9] and endo-β-(1→3)-glucanase from *Rhizopus arrhizus*[10].

Laminarinases III and IV were incubated with linear laminarin from *L. hyperborea* under standard conditions. An increase with time of the reducing power was

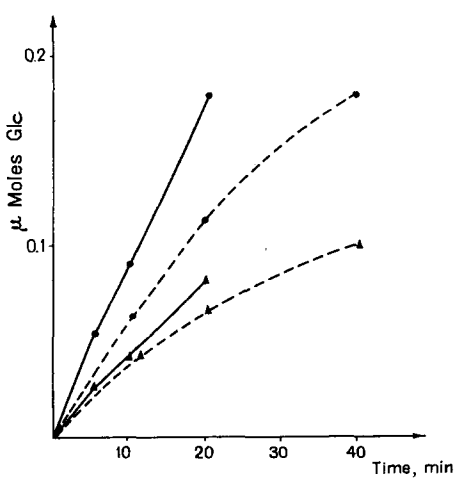

Fig. 1. Action of laminarinases III and IV on laminarin. — — —, laminarinase III; ———, laminarinase IV; ▲, release of glucose as determined by glucose oxidase method from laminarin; ●, release of reducing power as determined by the Nelson procedure on hydrolysis of laminarin with laminarinases III and IV.

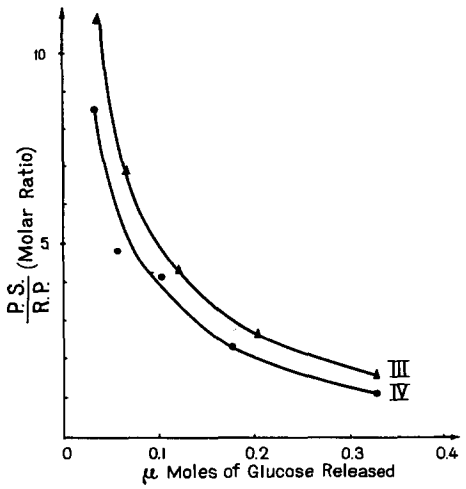

Fig. 2. Action pattern of the laminarinases from *S. sachalinensis* on laminarin. P.S., moles total carbohydrates as determined by phenol–H_2SO_4 method; R.P., reducing power expressed as glucose equivalent determined by the Nelson procedure.

observed using the Nelson method, and comparative determination of glucose liberated at hydrolysis was carried out by the glucose oxidase method. The experimental results are shown in Fig. 1.

On the other hand, we used the TUNG AND NORDIN[11] method to determine the action pattern of the glucanases. Laminarin from *L. hyberborea* (2 mg/ml) was incubated for 5 min at 37° with laminarinases III and IV dissolved consecutively. Fig. 2 illustrates the typical pattern.

We also undertook a time study of the action pattern of laminarinases III and IV upon laminarin from *L. cycharioides*. The obtained enzymolysis products (each aliquot containing a total of 2 mg of sugar) were subjected to gel filtration on Biogel

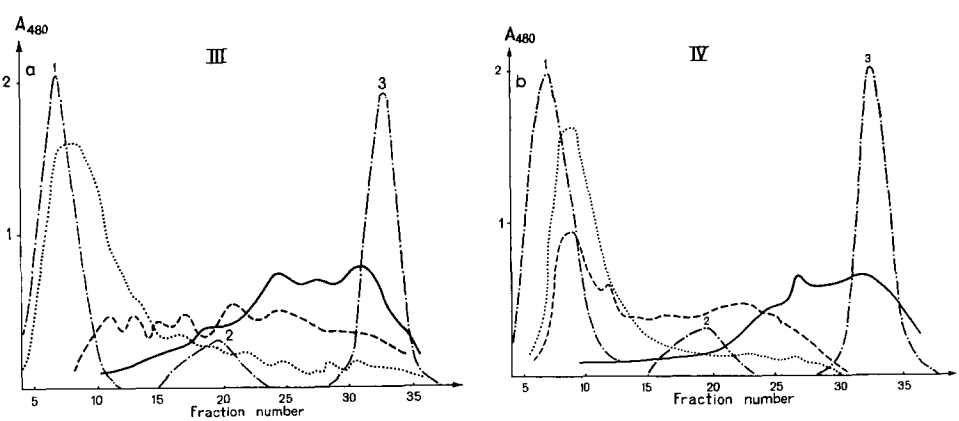

Fig. 3. Gel filtration of products of enzymatic hydrolysis of laminarin with laminarinases III (a) and IV (b), respectively, on Biogel P-2.
—·—, standards (1, initial laminarin; 2, laminaritetraose; 3, glucose). Incubation mixture: in 15 min (·····); in 30 min (— — —); and in 2 h (———).

TABLE V

RELATIVE VELOCITIES OF HYDROLYSIS OF MODIFIED LAMINARINS WITH LAMINARINASES III AND IV

Substrate	*Relative velocity*	
	Laminarinase III	*Laminarinase IV*
Control	(100)	(100)
Periodate-oxidized laminarin	59	105
Periodate-oxidized and reduced laminarin	57	105

P-2 (1.5 cm × 40 cm column; elution rate, 0.2 ml/min; fractions of 1 ml each). The carbohydrate content in the fractions was estimated by the phenol–H_2SO_4 method. The data obtained are given in Figs. 3a and 3b. Similar data for exo-laminarinase from *Basidiomycete QM 806* were previously cited by BOCHKOV *et al.*[12].

The anomeric configuration of sugars obtained with enzymatic hydrolysis of laminarin from *L. cycharioides* was determined by NMR spectroscopy. Laminarinases III and IV yielded products in which the anomeric configuration of the substrate was retained. We plan to publish detailed results in a special communication.

Enzyme effect on chemically modified laminarins

We also prepared various derivatives of laminarin in which the reducing and/or non-reducing ends were altered. In each of these cases, the enzymatic reaction rate was compared with the rate of attack on laminarin under similar conditions. Laminarins with altered reducing ends were air-oxidized and reduced, and laminarins, in which both the reducing and non-reducing ends had been altered, were periodate-oxidized, and periodate-oxidized and reduced with $NaBH_4$. The alteration of the reducing end did not affect the enzymatic hydrolysis rate. Other results are given in Table V.

Effect of laminarinases III and IV on glucans containing both β-(1 → 3)- and β-(1 → 4)-linkages (oat and barley glucans)

All the final substrate concentrations were the same as determined by the phenol–H_2SO_4 method, and equal to 0.04%. 0.1 ml of the substrate, and 0.1 ml of the enzyme, in suitable concentration, were mixed with 0.3 ml of the acetate buffer (pH 5.6) and incubated for 15 min at 37°. Hydrolysis was terminated by adding 0.5 ml of the Nelson reagent. The results are cited in Table VI.

TABLE VI

COMPARISON OF VELOCITIES OF HYDROLYSIS OF LAMINARIN AND GLUCAN FROM BARLEY WITH LAMINARINASES III AND IV

Substrate	*Relative enzyme units*	*Time (min)*	*Reducing power*	
			Laminarinase III	*Laminarinase IV*
Laminarin from *L. cycharioides*	(1)	15	0.9	0.97
Barley glucan	2	15	0.9	
	4	15		0.112

Enzyme effect on other polysaccharides

Laminarinases III and IV do not hydrolyse nigeran (polysaccharide with α-(1→3)- and α-(1→4)-linkages), xylan from *N. vermiculare* (probably with β-(1→3)- and β-(1→4)-linkages), amylose, soluble starch and chondroitinsulphate. This complements data reported earlier[1].

DISCUSSION

As apparent from Table I, the rather high asparatic and glutamatic acid content and relatively low basic amino acid content are characteristic features of the amino acid composition of laminarinase IV. Similar amino acid composition was reported earlier for other carbohydrases[7,13,14].

Laminarinase IV is insensitive to the presence of metal ions; K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} have no effect at $1 \cdot 10^{-3}$ M. Pb^{2+} and Cu^{2+} of the same concentration caused partial inhibition of the enzyme (see Table II). Ag^+ and Hg^{2+} caused complete inhibition of the enzyme at $1 \cdot 10^{-4}$ M, and only partial inhibition at $1 \cdot 10^{-5}$ M. Sodium azide, an inhibitor of reactions catalysed by metals, did not cause the inhibition of laminarinase IV. EDTA does not affect activity at $1 \cdot 10^{-3}$ M and 15 min of preincubation, and causes practically complete inhibition of the enzyme at $1 \cdot 10^{-2}$ M and prolonged preincubation. Monoiodoacetic acid does not affect activity. At $1 \cdot 10^{-4}$ M, sodium *p*-chloromercuribenzoate causes complete loss of enzyme activity, while at $1 \cdot 10^{-5}$ M 84% of the activity is retained. Sodium lauryl sulphate causes enzyme inhibition. These facts show that metal and sulphydryl groups are not essential for laminarinase IV activity.

K_m values (Table III) for linear laminarin from *L. hyperborea* and laminarin from *L. cycharioides* were common for enzymes of this type of action. Thus, K_m for endo-β-1→3-glucanase[10] was $1.23 \cdot 10^{-4}$ M. According to HASEGAWA AND NORDIN[9], the slightly higher K_m value for α-1,3-endo-glucanase, equal to $4.6 \cdot 10^{-2}$ M, was due to the insolubility of the substrate.

It is clear from Table IV that the hydrolysis rate of laminaridextrins increased with chain length. This is in good agreement with the action of other endo-glucanases[10].

Usually two patterns of enzymatic attack on polysaccharides were considered, namely when exo-enzymes remove monosaccharide or disaccharide units from the non-reducing end, and when endo-enzymes attack the glycosidic bonds at random. Furthermore, in order to determine the type of glucanase action, we used a number of tests elaborated previously[4,7–9,15].

Incipient data on the type of enzymatic action was obtained on comparing simultaneously measured quantities of all the reducing sugars and glucose formed at the initial stages of the reaction. As apparent from Fig. 1, the number of reducing sugars formed considerably surpassed the quantity of liberated glucose. Such a pattern indicates a probable endo-action of enzymes from *S. sachalinensis*.

When using the independent method[11] to determine the type of glucanase action, a pattern typical for endo-enzymes (Fig. 2) was obtained.

Gel filtration of the enzymatic hydrolysis products of laminarin with laminarinases III and IV on Biogel P-2 in time (Fig. 3) confirmed previous information on the endo-type of enzyme action. Indeed, a great number of oligosaccharides are

apparent at the 30th min of hydrolysis at very low monomer glucose content, and a sharp decrease of the peak height corresponding to polymer laminarin. Under similar conditions, we obtained the same pattern for endo-laminarinase from *R. arrhizus*.

It has been previously shown[16] that endo-polysaccharases give products which retain an anomeric configuration; at the same time, exo-polysaccharases cause an inverted anomeric configuration. Laminarinases III and IV undoubtedly belong to the first type; this was established by NMR spectroscopy of the enzymatic hydrolysis products.

In the case of glucanase action according to the exo-mechanism, modification of the terminal glucose unit of the non-reducing end causes an abrupt fall in enzyme activity[4]. For the endo-enzyme, such a modification has practically no effect upon its activity[9,10]. Laminarinases III and IV proved capable of hydrolyzing (see RESULTS) chemically modified substrates at the same or slightly lower rate. This is characteristic for endo-enzymes, for which the site of the initial attack lies outside the modification area.

Glucans with a mixed type of linkage (1→3) and (1→4) are subjected to the effect of endo-, and not exo-enzymes[4,9,10]. It is common knowledge that oat and barley glucans contain both β-(1→3)- and β-(1→4)-linkages, and investigation results showed that both these polysaccharides were subjected to the effect of laminarinases III and IV. It is noteworthy that laminarinase III hydrolyzed these glucans two times less effectively than laminarin, while the hydrolyzation power of laminarinase IV was 30 times weaker (see Table VI).

NELSON *et al.*[8] have previously shown that, compared to laminarin, exo-laminarinase attacks linear β-(1→3)- xylan at the rate of 1/30. On the other hand, endo-laminarinase does not hydrolyze the latter at all[10]. Now, laminarinases III and IV do not hydrolyze xylan from *N. vermiculare*.

Consequently, we are apt to think that both laminarinases from *S. sachalinensis* are endo-β-(1→3)-glucan glucanohydrolases. They specifically hydrolyze β-(1→3)-linkages in glucans, and are definitely inactive to α-(1→3)- and other types of linkages.

At the same time, we noticed some differences in the action patterns of both endo-laminarinases from *S. sachalinensis* in respect to each other and with endo-β-(1→3)-laminarinase from *R. arrhizus*, a sample of which was kindly supplied by Prof. S. Kirkwood. Thus, for laminarinases from *S. sachalinensis*, the ratio of all the reducing sugars to the amount of glucose liberated on hydrolysis was approximately equal to 2 (Fig. 1); for endo-laminarinase from *R. arrhizus*, it was approximately equal to 4 at the initial stages of hydrolysis. These discrepancies, as well as the above-mentioned difference in the hydrolysis rate of mixed glucans with laminarinases III and IV, were probably due to the effect on the type of enzyme action of a wide range of phenomena, previously mentioned by TOMA *et al.*[17]. These can possibly be differences in number and heterogeneity of the subsites, different transferase activity of the endo-enzymes, *etc.*

Further investigations are now under way.

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