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β-1,3-OLIGOGLUCAN:ORTHOPHOSPHATE GLUCOSYLTRANSFERASES FROM *EUGLENA GRACILIS*

I. ISOLATION AND SOME PROPERTIES OF A β-1,3-OLIGOGLUCAN PHOSPHORYLASE

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SUMMARY

1. A new enzyme has been found in cell free extracts of *Euglena gracilis* which catalyzes the reversible phosphorolytic cleavage of β-1,3-oligoglucans according to the following reaction:

$$(\beta\text{-1,3-glucose})_n + P_i \rightleftharpoons (\beta\text{-1,3-glucose})_{n-1} + \alpha\text{-D-glucose 1-phosphate}$$

This enzyme was distinguished from laminaribiose phosphorylase, which catalyzes the same reaction. They could also be separated using calcium phosphate gel.

2. In the direction of synthesis, glucose could serve as good an acceptor as β-1,3-oligoglucans but with a K_m approx. 10–20 times higher. Other β-glucosyl derivatives were also good acceptors, with the exception of laminarin and paramylon. On the other hand, several sugar phosphates could not substitute for α-glucose 1-phosphate.

3. The novel enzyme showed a nearly absolute requirement for sulfhydryl groups, a property especially observed in aged preparations.

4. The methods used for the identification of laminaritriose, one of the products formed using laminaribiose as acceptor, showed that the glucosyl moiety of α-glucose 1-phosphate was attached to the non-reducing end of the disaccharide.

5. Some kinetic properties of the enzyme and the stoichiometry of the reaction are also reported.

INTRODUCTION

A previous paper from this laboratory[1] dealt with laminaribiose phosphorylase, an enzyme isolated from *Euglena gracilis* strain z which catalyzes the reaction:

$$L_n + P_i \rightleftharpoons L_{n-1} + \alpha\text{-D-Glc-1-}P \qquad (1)$$

where L_n represents a β-1,3-oligoglucan including laminaribiose.

Further work has shown that extracts of the same organism contain another enzyme which catalyzes the same reaction but differs in several properties. As will be reported in the following paper[2], both enzymes can be separated by column chromatography. The new enzyme phosphorolyzes laminaritriose and higher homologues at a greater rate than laminaribiose, while the opposite behavior is observed with laminaribiose phosphorylase. Furthermore, the two enzymes differ in their requirement for compounds with –SH groups; this effect is especially observed in aged enzyme preparations. The new enzyme shows an absolute requirement for –SH bearing compounds while laminaribiose phosphorylase is only slightly activated.

The name β-1,3-oligoglucan:orthophosphate glucosyltransferase II is proposed for the new enzyme and β-1,3-oligoglucan:orthophosphate glucosyltransferase I for the previously reported laminaribiose phosphorylase. The trivial name β-1,3-oligoglucan phosphorylase for the new enzyme is used in this and in the following paper[2].

The present communication reports the isolation of the β-1,3-oligoglucan phosphorylase, its specificity and some of its kinetic parameters. The next paper[2] deals with comparative studies of the two phosphorylases.

EXPERIMENTAL PROCEDURE

Methods

P_i was determined by the method of FISKE AND SUBBAROW[3] and Glc-1-*P* either by the measurement of labile P_i (0.5 M H_2SO_4 for 5 min at 100°) or with the phosphoglucomutase–Glc-6-*P* dehydrogenase–NADP system[4].

Reducing power was measured according to SOMOGYI[5] and NELSON[6]; hexose was estimated by the phenol–H_2SO_4 method[7]. Degree of polymerization of oligosaccharides was determined as described by PEAT, WHELAN AND ROBERTS[8]. Glucose was determined with glucose oxidase and peroxidase but substituting 0.4 M Tris–HCl buffer, pH 7.6, for 0.1 M acetate buffer, pH 5.4 (ref. 9) or by the procedure of CAPUTTO, LELOIR AND TRUCCO[10] which minimizes interferences with reducing oligosaccharides.

Protein was estimated by the method of LOWRY *et al.*[11].

Chromatography was carried out on Whatman No. 1 or washed Whatman No. 3 MM paper. When the latter was used, a piece of Whatman No. 1 paper was sewed at the top of the sheet.

The solvent systems used were: (1) butanol–pyridine–water (6:4:3, by vol.); (2) propanol–ethyl acetate–water (7:1:2, by vol.).

Previous to the chromatographic runs, the enzymatic reaction products were desalted by passing through a column of Amberlite mixed-bed resin (acetate form) and the percolate was concentrated either in a vacuum or on a boiling-water bath.

Paper electrophoresis of sugars was performed on Whatman No. 1 paper with 0.05 M potassium borate for 2 h at 20 V/cm or with 2% sodium molybdate, pH 5.1, as described by BOURNE, HUTSON AND WEIGEL[12], for 75 min at 25 V/cm. Sugar spots were visualized according to TREVELYAN, PROCTER AND HARRISON[13].

Materials

The materials were the same as in the earlier paper[1] except for those listed below.

Calcium phosphate gel (20 g dry weight per l) was prepared according to KEILIN AND HARTREE[14].

Preparation of glycosides. Laminaribiosyl- and laminaritriosyl-hydroquinone were prepared by incubation of the enzyme preparation ("33–66% ammonium sulfate step", see below) with 20 mM arbutin, 20 mM Glc-1-*P*, 40 mM imidazole buffer, pH 6.5, and 1 mM EDTA for 1 h. After desalting as described above, the hydroquinone glycosides were isolated by chromatography with Solvent 1 and on washed Whatman No. 17 paper sewed to Whatman No. 1 paper.

Laminaribiosyl-, laminaritriosyl- and laminaritetraosyl-saligenin were prepared and isolated in the same manner but using salicin instead of arbutin in the incubation mixture.

The hydroquinone derivatives were identified as described before[1] and those corresponding to saligenin were tentatively recognized as follows. Laminaritriosyl- and laminaribiosyl-saligenin were partially hydrolyzed with 0.5 M acetic acid during 4 h, chromatographed on Whatman No. 1 paper in Solvent 1 and revealed with $AgNO_3$. Three and two spots were respectively detected which corresponded to the standards of laminaritriose, laminaribiose and glucose in the first case, and laminaribiose and glucose in the second. In addition, the R_F of the products and those of the original phenolic glucosides were determined in Solvents 1 and 2. A plot of log $(1/R_F - 1)$ against the number of hexose units[15] gave a linear relationship, indicating that the compounds belonged to a homologous series. This was the only criterion used to identify laminaritetraosyl-saligenin due to scarcity of material.

Enzyme assay

Two assays were used to determine the β-1,3-oligoglucan phosphorylase reaction. Procedure A was used to determine the enzyme activity on reaction (1) from right to left and procedure B to measure the phosphorolytic activity.

Procedure A

(1) Preincubation mixture. 2 μmoles of imidazol buffer, pH 7.2, 0.2 μmole of EDTA, 1 μmole of mercaptoethanol and enzyme (0.01 to 0.04 unit, see below), in a volume of 0.03 ml, were preincubated at 30° for 30 min.

(2) Incubation mixture. Laminaribiose* (0.5 μmole) and 1 μmole of Glc-1-*P* (dipotassium salt) were added to the preincubation mixture and incubated at 37° for 10 min; the final vol. was 0.05 ml. The reaction was stopped by 40-fold dilution with water and the released P_i was determined. Controls were prepared similarly but omitting the acceptor. This procedure was used in most experiments of the present study.

Procedure B

(1) Preincubation mixture. It was similar to that described above except that 2 μmoles of potassium phosphate buffer, pH 7.2, was used instead of imidazole buffer and that the amount of enzyme was lowered to 0.005–0.02 units.

(2) Incubation mixture. Laminaritriose (0.5 μmole) was added to the preincubation mixture and after 10 min of incubation at 37°, the Glc-1-*P* formed was

* The same initial reaction rate was obtained when 5 μmoles of glucose were substituted for laminaribiose.

determined, after heating 2 min at 100°, by the phosphoglucomutase–Glc-6-*P* dehydrogenase–NADP system.

Controls were carried out similarly but omitting the trisaccharide.

One unit of enzyme is defined as that amount catalyzing the formation of 1 μmole of P_i per min under the conditions described in *Procedure A*.

RESULTS

Crude extracts

Cells of *Euglena gracilis* strain z were grown under natural light at room temperature (24–30°)* and processed as described previously[1] up to the "33–66% ammonium sulfate step". Table I summarizes the results obtained in a typical experiment, starting from 1 l of culture medium (approx. 15 ml of green wet cells).

Adsorption on and elution from the calcium phosphate gel

The ammonium sulfate fraction (12 ml) was treated with enough calcium phosphate gel (5 mg of the gel/mg protein) to adsorb most of the protein. After standing

TABLE I

ENZYME PURIFICATION

The enzyme activity was determined as described under *Enzyme assay, Procedure A*.

Step	*Volume (ml)*	*Protein (mg/ml)*	*Activity (units/ml)*		*Specific activity (units/mg)*		*Yield (%)*		*Ratio glucose/laminaribiose*
			Laminaribiose	*Glucose**	*Laminaribiose*	*Glucose**	*Laminaribiose*	*Glucose**	
A Crude extract**	100	7.0	1.5	2.8	0.2	0.4	100	100	1.9
B Protamine sulfate supernatant**	100	3.0	1.5	2.8	0.5	0.9	100	100	1.9
C Ammonium sulfate 33–66%**	12	14.0	8.5	16	0.6	1.1	68	69	1.9
D Calcium phosphate gel eluate									
I 5 mM sodium pyrophosphate	4.4	3.1	0.7	15	0.2	4.8	1.9	23.5	22
II 5 mM sodium pyrophosphate	2.2	3.5	0.6	8	0.2	2.3	0.8	6.0	14
III 5 mM sodium pyrophosphate	2.2	4.0	5.6	6	1.6	1.5	8	4.7	1.0
IV 10 mM sodium pyrophosphate	3.6	5.2	9.2	9.5	1.8	1.8	22	11.8	1.0
V 10 mM sodium pyrophosphate	3.0	4.8	3.3	3.3	0.7	0.7	6.6	3.5	1.0
VI 20 mM sodium pyrophosphate	2.2	3.2	0.8	1.7	0.3	0.5	1	1.1	2.0

* Activity measured as described in *Enzyme assay, Procedure A*, but substituting 5 μmoles of glucose for laminaribiose. Under these conditions, both phosphorylases were actually measured in A, B, and C. Enzyme units defined as described in the text but with the mentioned change.

** Activity measured after dialysis against water.

* The same total or specific activity was obtained for both phosphorylases whether the cells were grown in the light or in the dark.

for 10 min in an ice bath, with occasional stirring, the mixture was centrifuged at 10 000 × *g* for 10 min and the enzyme was eluted from the gel with sodium pyrophosphate as described in Table I. At each step the suspension was allowed to stand in the cold, with occasional stirring, for 10 min and was centrifuged as above. All the eluates were separately dialyzed overnight against 1 l of 10 mM Tris buffer, (pH 7.2)–1 mM EDTA, with one change of the liquid.

Table I shows that Fractions I and II were enriched with laminaribiose phosphorylase while the new enzyme was eluted from the gel with 10 mM sodium pyrophosphate. Occasionally, however, β-1,3-oligoglucan phosphorylase appeared after the third elution with 5 mM sodium pyrophosphate.

In most of the experiments mentioned in this paper, Fraction IV was used as β-1,3-oligoglucan phosphorylase. The highest purification achieved was about 10-fold from the crude extract to the last fraction.

Comments

Table I shows that, under the described conditions, laminaribiose phosphorylase (Fractions I and II) catalyzed the P_i released from Glc-1-*P* at a rate 10–20-fold lower with laminaribiose than with glucose as acceptor. On the other hand, β-1,3-oligoglucan phosphorylase (Fractions III to V) liberated P_i from the phosphoric ester at similar rates using either the monosaccharide or the disaccharide as substrate (last column of Table I). These ratios of activities were consistently observed in different preparations, including in the enzymes separated by DEAE-cellulose column chromatography[2]. In most of the experiments described in the present paper, laminaribiose was used as acceptor. Thus, the possible contamination of the new enzyme with some laminaribiose phosphorylase cannot affect the results since, according to the amount of enzyme used, the P_i released due to the action of the disaccharide phosphorylase is within the experimental error. Obviously, as can be seen also in Table I, this reasoning cannot be applied when glucose was the acceptor, due to the fact that both enzymes act on the later sugar with similar velocities.

Stability

The stability was somewhat variable in different batches. 2- and 8-week-old extracts showed an apparent loss of activity of 80 and over 95%, respectively, when assayed without mercaptoethanol during the preincubation. When the latter step was carried out with –SH containing compounds (see *Enzyme assay*), about 50% of the original activity was recovered in 8-week-old preparations.

Kinetics

A linear relationship was obtained between the P_i released and time or enzyme concentration when the activity of β-1,3-oligoglucan phosphorylase was measured as described under *Enzyme assay, Procedure A*.

Table II shows the K_m and $v_{max.}$ of various acceptors. It can be seen that while the $v_{max.}$ was similar for all of the sugars tested, the K_m for glucose was about 10–20-fold higher than for β-1,3-oligoglucans. The K_m found for glucose was approx. twice that determined with laminaribiose phosphorylase, but at variance with the latter[2], higher concentrations of glucose were not inhibitory.

The K_m for P_i, assayed in the presence of 2 μmoles of imidazole buffer, pH 7.2,

TABLE II

KINETIC PARAMETERS

The incubation conditions were as described under *Enzyme assay, Procedure A*, but with the sugars indicated below and 0.01 ml of Fraction IV (see Table I). In all cases, initial reaction rates were used to determine the K_m.

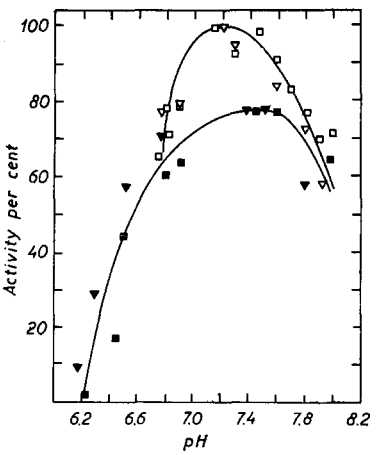
Substrate	K_m *(mM)*	$v_{max.}$ *(mμmoles/min)*
Glucose	40	29
Laminaribiose	4	38
Laminaritriose	4.5	35
Laminaritetraose	3	35
Laminaripentaose	2	25

was found to be 3 and 2 mM with laminaritetraose (13 mM) or laminaritriose (10 mM) as glucosyl donor, respectively; that of Glc-1-*P* was 1.8 mM when laminaribiose (10 mM) was used as acceptor.

pH optimum

Fig. 1 shows the effect of pH on the reaction rate with glucose or laminaribiose as acceptor. The optimal pH obtained with both substrates ranged from 7 to 7.5.

The effect of pH on the initial velocity when laminaritetraose, laminaritriose and laminaribiose were used as glucosyl donors to form Glc-1-*P* is shown in Fig. 2.



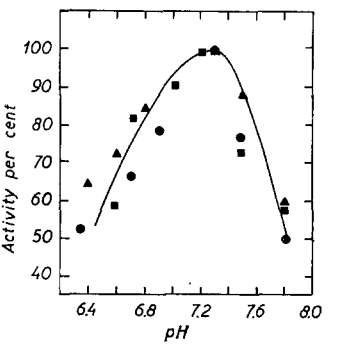


Fig. 1. pH activity curves with glucose or laminaribiose as acceptor. The conditions were as described under *Enzyme assay, Procedure A*, but using 5 μmoles of glucose (▼—▼, ▽—▽) or 0.7 μmole of laminaribiose (■—■, □—□). Full symbols: 40 mM Tris–maleate buffer; open symbols: 40 mM imidazole buffer. The results are expressed as % of maximal activity for each acceptor.

Fig. 2. The effect of pH on the initial rate of phosphorolysis: (●—●) laminaritetraose (0.5 μmole); (▲—▲) laminaritriose (0.5 μmole) and (■—■) laminaribiose (0.7 μmole). The conditions of incubation and assay were as described under *Enzyme assay, Procedure B*, except that the pH was changed as indicated and that 0.008 unit of enzyme was used for laminaritetraose and laminaritriose and 0.05 unit for laminaribiose. The results are expressed as in Fig. 1.

The highest activity for the three substrates was obtained at about pH 7.2. It should be noted that the reaction is slower with laminaribiose than with laminaritriose and laminaritetraose, so that six times as much enzyme had to be used in order to obtain similar amounts of Glc-1-*P*. Two possibilities may explain this result: the first is that the preparation of β-1,3-oligoglucan phosphorylase was contaminated with some laminaribiose phosphorylase. The other is that the reaction rate of the former enzyme with laminaribiose is about 6-fold lower than with the other oligosaccharides. Although at this time it is not easy to discard the possibility of contamination, the results presented in the next paper[2] support the second explanation.

On the other hand, and although this is not conclusive evidence, the fact that the activity profile as a function of pH was the same for the three sugars, suggests that only one enzyme is involved in the reaction.

Reaction product

When β-1,3-oligoglucan phosphorylase was incubated for 15 min or more under standard conditions with laminaribiose as acceptor, a series of reducing substances with R_F corresponding to those of laminaritriose and higher oligosaccharides, up to at least laminariheptaose, as well as glucose, were detected by paper chromatography. A similar pattern was observed when 10 or 20 mM glucose was substituted for laminaribiose.

In order to identify the first higher homologue of laminaribiose formed by enzyme action, the following steps were carried out (see Fig. 3).

Step 1: Obtaining ^{14}C-labeled trisaccharide. A twice scaled up standard mixture

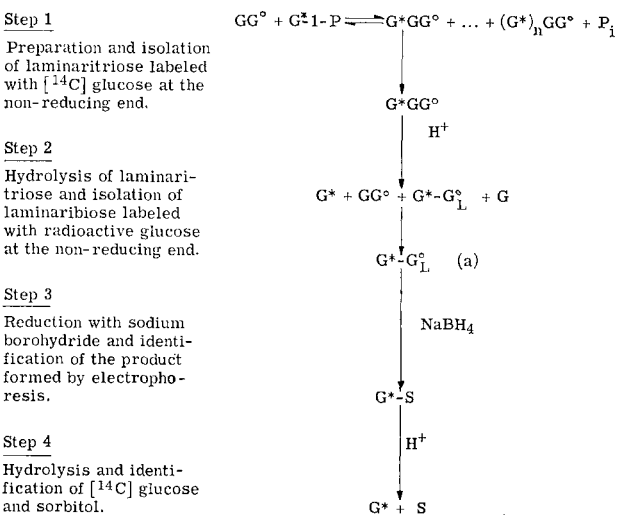


Fig. 3. Scheme of laminaritriose identification. For details see text. G: glucose; G°: reducing end glucose; G*: ^{14}C-labeled glucose; G^0_L: reducing end glucose originated from the non-reducing end of laminaribiose; S: sorbitol; G*GG° and $(G^*)_nGG°$: trisaccharide and undetermined β-1,3-glucosyloligosaccharide, respectively, formed by enzymic action; G*-1-*P*: [^{14}C]Glc-1-*P*. (a) The unlabeled disaccharide originated from the radioactive trisaccharide was not taken into account because it did not interfere in the identification.

was prepared and preincubated as under *Enzyme assay, Procedure A*. Then 5 μmoles of laminaribiose and 5.3 μmoles of [^{14}C]Glc-1-*P* (26 400 counts/min per μmole) were added in a final vol. of 0.1 ml and incubated at 37° for 1 h. The reaction was stopped by heating and after desalting and concentrating as described under *Methods*, the percolate was spotted on Whatman No. 3 MM paper and chromatographed with Solvent 1 during 20 h. Then the paper was dried and rechromatographed in the same solvent for another 20 h. A scanning for radioactivity was made and the area corresponding to the trisaccharide was cut out and eluted with water. Approx. 0.8 μmole of the sugar with a specific activity of 25 000 counts/min per μmole was obtained.

Step 2: Obtaining of ^{14}C-labeled disaccharide. Approximately one half of the ^{14}C-labeled sugar obtained in the previous step was diluted with 2 μmoles of cold laminaritriose and submitted to a partial hydrolysis in 0.165 M H_2SO_4 for 1 h at 100°. The hydrolysate was neutralized with $Ba(OH)_2$. The $BaSO_4$ formed was removed by centrifugation and discarded after washing with water.

The supernatant fluid and the washings were pooled, concentrated, spotted on Whatman No. 3 MM paper, and chromatographed for 40 h in Solvent 1.

After automatic scanning for radioactivity, the area corresponding to the ^{14}C-labeled disaccharide was cut out and eluted with water.

Step 3: Reduction of labeled disaccharide. A carrier of unlabeled laminaribiose (2 μmoles) was mixed with the radioactive disaccharide, and sodium borohydride, in a final concn. of 0.7% was added. The mixture was allowed to stand for 1 h at room temperature and the remaining borohydride was decomposed by adding two drops of 5 M HCl. The mixture was then evaporated 3 or 4 times in a vacuum, in the presence of methanol. After desalting, the reduced ^{14}C-labeled disaccharide (laminaribiitol (G-S)) was submitted to paper electrophoresis in sodium molybdate as described in *Methods*. The scanning for radioactivity showed only one peak with a $M_{sorbitol} < 0.1$. Under the described conditions, sorbitol ran about 17 cm; no correction was made for electro-osmosis. According to BOURNE, HUTSON AND WEIGEL[16], 2-, 4- and 6-O-substituted D-glucitol will form a complex with molybdate while the 3-O-substituted isomer will not. Hence, only the latter derivative will remain immobile by ionophoresis in molybdate. This result permits one to assign to the reduced disaccharide a 1,3-bond.

Step 4: Hydrolysis of the reduced disaccharide. In order to confirm that the product formed in Step 3 was laminaribiitol, the radioactive band of the ionophoreto gram was eluted with water and desalted as described before. The percolate was hydrolyzed in 1 M HCl at 100° for 1 h. Subsequently, it was dried under vacuum, neutralized with pyridine and again run in electrophoresis as mentioned in Step 3. By scanning, a radioactive peak with a $M_{sorbitol} < 0.1$ was again observed. This zone was cut out and eluted with water. The rest of the paper strip was visualized according to TREVELYAN, PROCTER AND HARRISON[13] and a spot running as sorbitol was detected. The eluted radioactive material was desalted as above and then submitted to paper electrophoresis in sodium tetraborate. An automatic scanning of the paper strip showed a radioactive band with a $M_{glucose}$ of 1.

As mentioned before, these results demonstrate the existence of a 1,3-bond between the two non-reducing glucoses of the trisaccharide. As the latter was obtained from laminaribiose, it can be designated as a glucosyl-1,3-glucosyl β-1,3-glucose.

TABLE III

IDENTIFICATION OF LAMINARITRIOSE

The trisaccharide was prepared and isolated as described in the text. The chromatographic paper eluate was concentrated in vacuum and aliquots were taken to carry out the determinations described below.

Total hexose (μmoles glucose/ml)		*Reducing power (μmoles glucose/ml)*	*Ratio total hexose before reduction/ total hexose after reduction*	*Ratio total hexose before reduction/ reducing power*	*Degree of polymerization***	*Total glucose/ ml****
*Before reduction**	*After reduction**					
15	10	4.2	1.5	3.6	3	15

* With sodium borohydride[8].

** Determined as described by PEAT, WHELAN AND ROBERTS[8].

*** Determined after β-glucosidase and glucose oxidase–peroxidase action. An aliquot of the product was taken and incubated with β-glucosidase (1.6 mg/ml) and 0.1 M acetate buffer, pH 5.1, during 2 h. The reaction was stopped by heating and the glucose liberated was determined with the glucose oxidase–peroxidase system in the presence of 0.4 M Tris buffer, pH 7.2.

In order to complete the identification, the trisaccharide was formed enzymatically as mentioned above but using cold Glc-1-*P*. After isolation by paper chromatography on Whatman No. 3 MM paper in Solvent 1, the determinations shown in Table III were carried out.

From the data obtained it could be concluded that laminaritriose was formed

TABLE IV

STOICHIOMETRY

In Expt. 1 the preincubation mixture (Fraction IV as enzyme) was as described under *Enzyme assay, Procedure A*, but the total amount was increased 3-fold. After 30 min at 30°, laminaribiose labeled with ^{14}C in the non-reducing end (2.1 μmoles, 25 200 counts/min) and Glc-1-*P* (3 μmoles) were added in a final vol. of 0.15 ml, and incubated for 10 min at 37°. The reaction was stopped by heat and aliquots were taken to assay the P_i liberated and the remaining Glc-1-*P*. The latter was determined as acid-labile phosphate. Another aliquot (0.05 ml) was spotted on Whatman No. 1 paper and chromatographed for 40 h in Solvent 1. The chromatograms were cut in 1-cm horizontal segments and counted in a Frieseke windowless gas-flow counter. The radioactivity of the different compounds was determined. Control tubes were run in a similar way but Glc-1-*P* was added at the end of the incubation. In Expt. 2, the conditions were similar except that 1.9 μmole of laminaribiose (45 000 counts/min) was added and the tubes were incubated for 80 min at 37°. The radioactivity of the different compounds was estimated as above but using a Picker automatic thin window flow counter. The values given correspond to 0.05 ml of incubation mixture.

Expt. No.	*Substrate disappearance (μmoles)*		*Product formed (μmoles)*				
	Glc-1-P	*Laminaribiose*	P_i	*Laminaritriose*	*Laminaritetraose*	*Laminaripentaose*	*Laminarihexaose*
1	0.37	0.29	0.37	0.21 (0.21)	0.06 (0.12)	0.01 (0.03)	Undetected
2	0.68	0.41	0.68	0.19 (0.19)	0.10 (0.20)	0.08 (0.24)	0.02 (0.08)

by the action of β-1,3-oligoglucan phosphorylase. The higher oligosaccharides formed by the latter enzyme were identified as laminaritetraose, laminaripentaose, laminarihexaose and laminariheptaose, by comparison with a paramylon hydrolysate in paper chromatography with Solvents 1 and 2.

Moreover, a plot of $-\log R_G$ against the number of glucose units according to JOHNSTON[17] gives a linear relationship, thus indicating that they were oligosaccharides of the same β-1,3 series.

Stoichiometry

Table IV shows the stoichiometry of the reaction. It can be observed that the disappearance of Glc-1-*P* and laminaribiose agree with the formation of P_i and higher oligosaccharides, respectively. In Expt. 1 (10 min incubation), a small but significant amount of laminaripentaose was formed while in Expt. 2 (80 min) laminarihexaose could also be detected. Values in parentheses correspond to μmoles of glucosyl residues originated from Glc-1-*P*. A good agreement between the sum of these values and that of P_i liberation can be seen.

TABLE V

ACCEPTOR SPECIFICITY

Assay mixtures were prepared as described in text with laminaribiose or other acceptors, at 10 mM concentration, and 15 min incubation at 37°. The values obtained with laminaribiose (0.32 μmole) were taken as 100%.

Substrate	*P_i liberated (%)*
Laminaribiose	100
Laminaritriose	100
Laminaribiosyl-β-1,4 glucose	100
Laminaritriosyl-*p*-hydroquinone	100
Laminaritriosyl-saligenin	100
Arbutin	98
Salicin	98
Laminaritetraosyl-saligenin	94
Laminaritetraose	85
Laminaribiosyl-*p*-hydroquinone	85
β-Phenylglucoside	78
β-Methylglucoside	75
Cellobiose	72
Laminaripentaose	66
Laminaribiosyl-saligenin	63
Laminaridextrins*	56
Laminariheptaose	51
Glucose	44
Gentiobiose	37
α-Phenylglucoside	6
KOH-treated paramylon (1 mg)	<5
Laminarin (1 mg)	<5
Maltose, L-xylose, D-xylose, raffinose, sucrose, isomaltose, melibiose, sorbitol, 2-deoxyglucose, trehalose, fructose, and α-methylglucoside	<5

* Mixture of laminaridextrins, probably laminaripentaose to laminariheptaose, 70 mM, expressed as glucosyl units.

Specificity

α-Glc-1-*P* is quite specific as donor of the glucose moiety; the following sugar phosphates were ineffective: α-Man-1-*P*, α-Gal-1-*P*, β-Glc-1-*P* and Fru-1,6-P_2. On the other hand, besides glucose, several β-substituted glucosides may act as acceptors, as shown in Table V.

By comparison of the results shown in the table with those previously reported for laminaribiose phosphorylase[1], one can observe a qualitative similarity in acceptor specificity. However, the enzymes differ from the quantitative point of view. Thus, when the rates of P_i released by laminaribiose phosphorylase using glucose as acceptor were taken as 100%, the values for most of the other sugars was below 25%.

Arsenolysis

When β-1,3-oligoglucan phosphorylase was incubated with arsenate and β-1,3-glucosyl oligosaccharides, a cleavage of the sugars was detected. The values corresponding to the reducing power liberated after 20 min incubation, expressed as glucose, are shown in Table VI. No glucose could be detected when KOH-treated paramylon[18] or laminarin was used as substrate. This result together with the fact

TABLE VI

ARSENOLYSIS

The preincubation mixture was as follows: 2 μmoles of imidazole buffer, pH 7.2; 0.2 μmole of EDTA, 1 μmole of mercaptoethanol and 0.01 ml of Fraction IV (see Table I) in a total vol. of 0.03 ml. After 30 min preincubation at 30°, the substrates in the concentrations indicated below and 1 μmole of potassium arsenate, pH 7.2, in a final vol. of 0.05 ml were added and incubated at 37° for 20 min. Reducing power was determined by the method of CAPUTTO, LELOIR AND TRUCCO[10]. Controls were carried out by addition of substrates at the end of the incubation.

Substrate	*Concentration (mM)*	*Glucose liberated (μmoles)*
Laminaribiose	10	0.29
Laminaritriose	10	0.44
Laminaritetraose	10	0.40
Laminaripentaose	10	0.48
Laminarihexaose	7	0.30
Laminaridextrins	70 (as glucose)	0.44
KOH-treated paramylon, laminarin	2 mg	0
Glc-1-*P*	20	0

that the polysaccharide did not serve as acceptor (Table V) indicates a certain specificity towards the glucosyl chain length.

On the other hand, no arsenolysis of Glc-1-*P* could be observed in the absence of acceptor. This behavior is similar to that of maltose, cellobiose[19] and laminaribiose phosphorylases[1]. This finding might indicate that no glucosyl–enzyme intermediate is formed and that the reaction occurs by a single displacement mechanism.

Requirement for –SH groups

Fig. 4 shows the dependence of the reaction rate on the concentration of mercaptoethanol. It can be observed that the optimal concn. is about 20 mM and

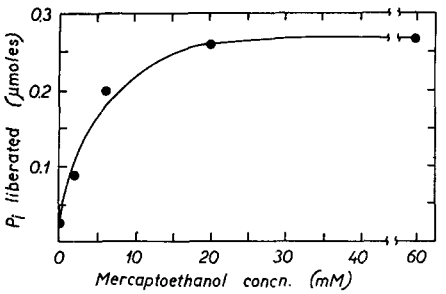


Fig. 4. Effect of mercaptoethanol concentration on the reaction rate. Conditions as described under *Enzyme assay, Procedure A*, except for the concentrations of mercaptoethanol.

that the difference between the presence and absence of mercaptoethanol was about 13-fold. This difference was usually obtained with enzymes about 2 months old. In addition, glutathione and cysteine, at a concn. of 20 mM could reactivate the enzyme to the same extent as mercaptoethanol, while EDTA was ineffective. This finding would indicate that the enzyme reactivation was not due to the chelating effect of the sulfhydryl compounds, but to their reducing power.

On the other hand, the preincubation time required for optimal enzyme reactivation at 30° was approx. 15–30 min, at 20 mM of mercaptoethanol.

Inhibitors and activators

Neither activation nor inactivation was observed by adding the following compounds to the standard reaction mixtures: 2 mM UDP, UDP-Glc, CDP, ITP, ATP, ADP, sodium pyrophosphate, 5 mM AMP, 0.01 mM cyclic 3′,5′-AMP and 4 mM hydroquinone. However, when mercaptoethanol was omitted from the standard incubation mixture using fresh enzyme preparations, 4 mM hydroquinone inhibited 80% and 90% of the original activity with glucose or laminaribiose as acceptor, respectively.

When the enzyme was assayed in the direction of phosphorolysis, in the absence of EDTA, no variation in the formation of Glc-1-*P* was detected by adding 3 mM ATP, 2 mM UTP, 40 mM imidazole, 20 mM $MnCl_2$, 4 mM $MgCl_2$, 20 mM glucose, 20 mM sodium pyrophosphate, 1.8 mM UDP, 4 mM EDTA and 20 mM sorbitol; with 3 mM UMP an inhibition of about 30% was observed.

DISCUSSION

It appears from the data shown in Table I that the enzyme reported in this paper is clearly distinguishable from laminaribiose phosphorylase. The ratio of activities of the two enzymes remains rather constant during the three first steps of purification; but most of the laminaribiose phosphorylase is obtained in the fraction eluted with 5 mM sodium pyrophosphate, while a concn. of 10 mM is necessary to obtain the highest activity of β-1,3-oligoglucan phosphorylase. These data are in apparent contradiction with those reported previously by GOLDEMBERG, MARECHAL AND DE SOUZA[1], who found that the ratio of enzymic activity between laminaritriose and laminaribiose (Eqn. 1, left to right) as substrates remained quite constant during

the different steps of purification. Probably this was due to the fact that the assays were carried out with old enzyme preparation and in the absence of mercaptoethanol. As can be seen in Fig. 4 only a minute amount of P_i was liberated from Glc-1-*P* when β-1,3-oligoglucan phosphorylase was assayed in the absence of –SH donors.

It is difficult to ascertain the specificity of each enzyme. Both of them can use almost the same acceptors and form β-1,3-oligosaccharides from glucose and Glc-1-*P*, but they show different kinetic behavior. Thus β-1,3-oligoglucan phosphorylase forms higher oligosaccharides from 10 mM laminaribiose, laminaritriose and laminaritetraose, about twice as fast as from glucose at the same concentration (Table V). Laminaribiose phosphorylase, on the contrary, synthesizes the same oligosaccharides from 10 mM glucose at a 3–4-fold higher rate than from the di-, tri- and tetrasaccharide[1].

It remains to be determined whether laminaribiose phosphorylase can phosphorolyze laminaritriose or higher β-1,3-oligosaccharides, or whether the action was due to a contamination with β-1,3-oligoglucan phosphorylase. On the other hand, the latter enzyme phosphorolyzes laminaribiose but no definite proofs were obtained in this paper as to whether this effect is due to a contamination with laminaribiose phosphorylase, as might be suspected from the separation methods. The next paper deals with comparative studies on both enzymes in order to gain further insight into this problem.

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