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

II. COMPARATIVE STUDIES BETWEEN LAMINARIBIOSE- AND β-1,3-OLIGOGLUCAN PHOSPHORYLASE

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SUMMARY

1. Further studies have been carried out on the specificity of laminaribiose- and β-1,3-oligoglucan phosphorylase. Both enzymes, present in extracts of *Euglena gracilis*, could be separated either by calcium phosphate gel or by DEAE-cellulose column chromatography.

2. From the data presented it can be concluded that the two enzymes catalyze the same reaction but with different quantitative specificity. The β-1,3-oligoglucan phosphorylase was found to phosphorolyze laminaritriose and higher homologues at a greater rate than laminaribiose while the opposite behavior was observed with laminaribiose phosphorylase.

3. Another, and probably more striking, difference between the two enzymes was the fact that aged preparations of β-1,3-oligoglucan phosphorylase showed an absolute requirement of sulfhydryl donors while laminaribiose phosphorylase was only slightly activated.

4. The possibility that the qualitative similarity might be due to a cross-contamination of the enzymatic fractions was discarded by competition experiments between substrates and by the elution pattern of the DEAE-cellulose chromatography.

INTRODUCTION

Two separable enzyme activities on β-1,3-oligoglucans are present in extracts of *Euglena gracilis* strain z: laminaribiose phosphorylase (β-1,3-oligoglucan:orthophosphate glucosyltransferase I) and β-1,3-oligoglucan phosphorylase (β-1,3-oligoglucan:orthophosphate glucosyltransferase II). In the preceding communication[1], some properties of the latter enzyme were described, as well as its separation from laminaribiose phosphorylase.

Since both phosphorylases catalyze the same reaction,

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

a comparative study between both enzymes has now been carried out in order to gain further insight into their similarities and differences. In addition, a method for the separation of the two enzymes by DEAE-cellulose column chromatography is reported.

EXPERIMENTAL PROCEDURE

Most of the materials and methods used were the same as previously described[1] except for those specifically mentioned.

Enzyme assay

Unless otherwise specified they were as follows:

Laminaribiose phosphorylase. Both Procedures* A and B, detailed in the previous work, have been used except that 5 μmoles of glucose were used instead of laminaribiose in *Procedure A*, and 0.5 μmole of the latter sugar was substituted for laminaritriose in *Procedure B*. Controls were carried out similarly but omitting the sugars.

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***.

β-1,3-oligoglucan phosphorylase. Either Procedure A or B, described previously[1], was used to measure its activity.

RESULTS

Enzyme preparation from calcium phosphate gel

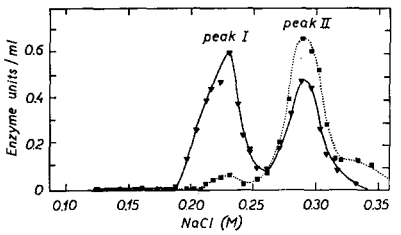
Cells of *Euglena gracilis* strain z were grown, harvested and processed as described in the preceding paper[1]. The first fraction eluted from the calcium phosphate gel with 5 mM sodium pyrophosphate (Fraction I, containing 0.01 to 0.04 units) was used as laminaribiose phosphorylase and the first fraction eluted with 10 mM sodium pyrophosphate (Fraction IV, containing 0.01 to 0.04 units) was employed as β-1,3-oligoglucan phosphorylase.

Enzyme preparation from DEAE-cellulose column chromatography

Cells of the same organism grown in darkness were harvested and processed as described previously[2] up to the 33–66% ammonium sulfate step. The latter fraction (13 ml, 50 mg protein per ml) was dialyzed overnight against 10 mM Tris–HCl–1 mM EDTA, pH 7.2, and then applied to a column of DEAE-cellulose (16 cm^2 × 32 cm) equilibrated with the same buffer. All operations were carried out at 0–4°. After the protein was passed through the column, the latter was washed with 100 ml of the same buffer, and then with 100 ml of 0.1 M NaCl in 10 mM Tris–HCl–1 mM

* The preincubation of laminaribiose phosphorylase with mercaptoethanol was not necessary for activity. However, it was carried out as usual in order to standardize the assay of both phosphorylases.

** This definition of laminaribiose phosphorylase unit is different from that given previously[2].



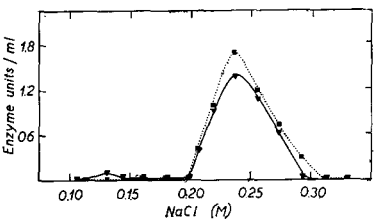


Fig. 1. Chromatography on DEAE-cellulose. 13 ml of the "33–66% ammonium sulfate step" fraction[2] was applied to a column (16 cm² × 32 cm) equilibrated with 0.01 M Tris–HCl–0.001 M EDTA, pH 7.2. A linear gradient of NaCl between 0.1 and 0.35 M in the equilibrium buffer was applied to the column (500 ml of each concentration) with a flow rate of 1–1.5 ml/min. Fractions of approx. 5 ml were collected. The enzyme activity was measured as described in *Enzyme assay, Procedure A*. Acceptor: (▼—▼) glucose; (■—■) laminaribiose.

Fig. 2. DEAE-cellulose column chromatography of Peak II. 12 ml of the protein extract from Peak II (see text) were passed through a 0.75 cm² × 32 cm column of DEAE-cellulose equilibrated with 0.01 M Tris–HCl–0.001 M EDTA, pH 7.2. A linear gradient of NaCl between 0.1 and 0.35 M in the equilibrium buffer (30 ml of each concentration) with a flow rate of 0.3 ml/min. Fractions of approx. 1.5 ml were collected. Symbols as in Fig. 1.

EDTA, pH 7.2. The protein was eluted with a linear gradient of NaCl (from 0.1 M to 0.35 M concentration in the equilibrium buffer, 500 ml of each saline concentration). The fractions containing the peaks of activity were pooled (Fig. 1) and 2.5 vol. of neutral saturated ammonium sulfate solution were added. The suspension was allowed to stand overnight before centrifugation at 10 000 × *g* for 20 min. The precipitate obtained was dissolved in a small volume of 10 mM Tris–HCl–1 mM EDTA buffer, pH 7.2, and dialyzed overnight against the same solution.

Fig. 1 shows that two similar activity peaks were obtained using glucose as substrate; one of them (Peak I, laminaribiose phosphorylase) was eluted between 0.18 and 0.23 M NaCl and the other (Peak II, β-1,3-oligoglucan phosphorylase) between 0.25 and 0.32 M NaCl. When laminaribiose was used as acceptor, two activity peaks were also obtained, but the activity of Peak I was very small compared with Peak II. It can also be observed that the profile of activities obtained with the two acceptors were coincident.

In order to discard the possibility that the presence of two activity peaks might be due to some artifact in the column chromatography, the protein fraction originated from Peak II was rechromatographed in a 0.75 cm² × 32 cm DEAE-cellulose column prepared as already mentioned. Fig. 2 shows that only one peak was obtained which emerged at approximately the same saline concentration as previously. It must be mentioned that when analytical DEAE-cellulose columns (0.75 cm² × 32 cm) were used, the laminaribiose phosphorylase was eluted between 0.14 and 0.19 M and the β-1,3-oligoglucan phosphorylase emerged between 0.22 and 0.26 M NaCl.

After the rechromatography of Peak II, the active fractions were pooled, concentrated and dialyzed as mentioned above. This preparation and that corresponding to Peak I were used as β-1,3-oligoglucan phosphorylase and laminaribiose phosphorylase, respectively, to carry out some of the assays mentioned below.

Comparative studies using enzymes separated with calcium phosphate gel

Effect of mercaptoethanol concentration on the reaction rate of both enzymes. The different behaviors of the phosphorylases towards increasing concentrations of

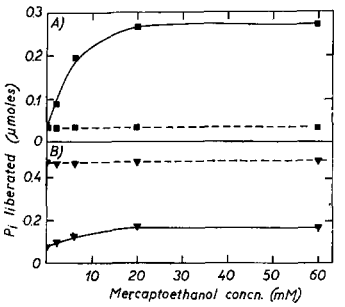


Fig. 3. Influence of mercaptoethanol concentration on the activity of β-1,3-oligoglucan and laminaribiose phosphorylase. (A) Laminaribiose (0.5 μmole) as acceptor. The standard assay for each enzyme was as described in *Procedure A*, except that the –SH donor concentration was varied as shown. For the assay of β-1,3-oligoglucan phosphorylase, Fraction IV was used and for that corresponding to laminaribiose phosphorylase, Fraction I was employed. (B) Glucose (5 μmoles) as acceptor. The assays were similar to (A) except that another enzyme batch was used, and that the third fraction eluted with 5 mM sodium pyrophosphate (Fraction III) was employed for the assay of β-1,3-oligoglucan phosphorylase. Symbols as in Fig. 1. Solid line: β-1,3-oligoglucan phosphorylase; broken line: laminaribiose phosphorylase.

mercaptoethanol using laminaribiose as acceptor is shown in Fig. 3A. It can be seen that the reaction rate for β-1,3-oligoglucan phosphorylase was increased about 10-fold at optimal concentration of –SH donor, while that for laminaribiose phosphorylase remained unchanged.

Similar effects were obtained when glucose was used as acceptor (Fig. 3B) but only a 2-fold increase in the reaction rate for β-1,3-oligoglucan phosphorylase was observed. This decrease in the activation might be due to a certain contamination of the latter enzyme preparation with laminaribiose phosphorylase, thus masking the exact amount of P_i released in absence of mercaptoethanol (see below).

Phosphorolysis of β-1,3-oligoglucans. Both enzymes catalyze the phosphorolysis of several β-1,3-oligoglucans, but the initial reaction rates were different.

As shown in Table I, laminaribiose phosphorylase acted on laminaribiose to form α-Glc-1-*P*, 3–4 times faster than on higher oligosaccharides. The inverse was true for β-1,3-oligoglucan phosphorylase.

It must be pointed out that laminariheptaose was also phosphorolyzed by laminaribiose phosphorylase. Thus, in an experiment similar to that described in

TABLE I

PHOSPHOROLYSIS OF β-1,3-OLIGOGLUCANS

The test conditions were as described in *Enzyme assay, Procedure B*. The oligosaccharides indicated below (0.5 μmole of each) were added and the amount of Glc-1-*P* formed was measured with the phosphoglucomutase–Glc-6-*P* dehydrogenase–NADP system.

Substrate	*Laminaribiose phosphorylase (mμmoles Glc-1-P formed per 10 min)*	*β-1,3-oligoglucan phosphorylase (mμmoles Glc-1-P formed per 10 min)*
Laminaripentaose	34	80
Laminaritetraose	44	80
Laminaritriose	46	80
Laminaribiose	170	20

Table I and taking the amount of Glc-1-*P* formed with laminaribiose as 100%, approx. 3% of the sugar phosphate came from 1 mM laminariheptaose. Under the same conditions, β-1,3-oligoglucan phosphorylase formed 130% of Glc-1-*P*.

Phosphorolysis of β-glucosyl derivatives of hydroquinone and saligenin by laminaribiose phosphorylase. It was previously reported[2] that two or more additional products were formed when laminaribiose phosphorylase was incubated with Glc-1-*P* and several acceptors, as judged by paper chromatography. However, in the case of arbutin, salicin and β-phenylglucoside only one additional spot was detected. It was suggested that another enzyme could be responsible for the different behavior of these acceptors. A further possibility could be that the higher homologues were not detected due to the scarcity of the material enzymatically produced. A new approach was then attempted taking advantage of the reversibility of the reaction and the higher sensibility of the method for Glc-1-*P* determination as compared to that for P_i. As shown in Table II, laminaritriosyl-hydroquinone, laminaritetraosyl-saligenin and laminaritriosyl-saligenin were phosphorolyzed by laminaribiose phosphorylase, but the reaction rate was about 90% lower than for the phenolic monosaccharides. For comparison, the values obtained with laminaribiose and laminaritetraose are also given. Although the results are not conclusive, the possibility remains that laminaribiose phosphorylase is actually involved in the reaction.

TABLE II

PHOSPHOROLYSIS OF VARIOUS SUBSTRATES WITH LAMINARIBIOSE PHOSPHORYLASE

The enzyme was assayed as described under *Procedure B*, except for the glucosyl donors that replace laminaribiose as detailed below. The Glc-1-*P* formed during the incubation was determined as described in Table I and the amount corresponding to laminaribiose was taken as 100% of relative activity.

Glucosyl donor	*Concentration (mM)*	*Relative activity*
Laminaribiose	10	100
Laminaribiosyl-hydroquinone	8	130
Laminaribiosyl-saligenin	8	135
Laminaritetraose	13	18
Laminaritriosyl-hydroquinone	8	10
Laminaritriosyl-saligenin	8	5
Laminaritetraosyl-saligenin	7	6

Glucose and several β-1,3-oligoglucans as acceptors for both enzymes. Since both enzymes could phosphorolyze the same β-1,3-oligo sugars, although at different rates, it was considered of interest to assay these compounds as acceptors. Fig. 4 shows that laminaribiose phosphorylase and β-1,3-oligoglucan phosphorylase can actually also catalyze at different rates similar reactions in the direction of synthesis.

Comparative studies using enzymes separated with DEAE-cellulose column chromatography

Specificity. β-1,3-oligoglucan phosphorylase originated from the rechromatography on DEAE-cellulose, was assayed with most of the acceptors previously used[1] and similar values for the liberation of P_i were obtained.

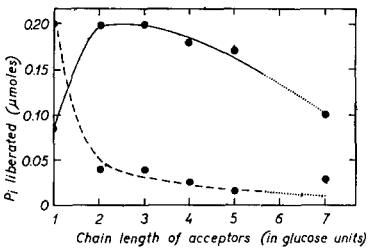
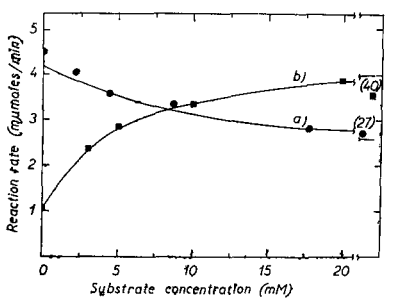


Fig. 4. Glucose and several β-1,3-oligoglucans as acceptors for laminaribiose and β-1,3-oligoglucan phosphorylases. The enzymatic assays were as described under *Procedure A* for the acceptors indicated. The concentration of the sugars was 5 mM, except for laminariheptaose which was 2.4 mM. After 15 min incubation, the P_i liberation was determined. Symbols and lines as in Fig. 3.

From the results of Figs. 1 and 2, it appears that both enzymes can use glucose and laminaribiose as acceptor. However to exclude the possibility of a mutual contamination, competition experiments between acceptors were performed as a new approach to solve this problem. The results are illustrated in Figs. 5 and 6. The theoretical curves were obtained using the following equations according to DIXON AND WEBB[3] for the determination of the total velocity (*vt*) when a single enzyme acts on two substrates *a* and *b*

$$vt = \frac{Va\frac{a}{K_a} + Vb\frac{b}{K_b}}{1 + a/K_a + b/K_b}$$



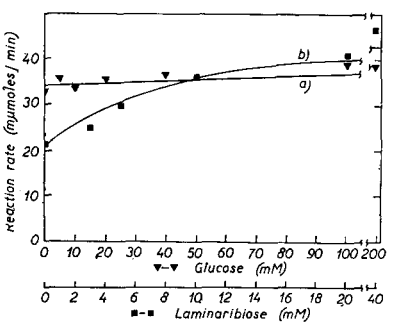
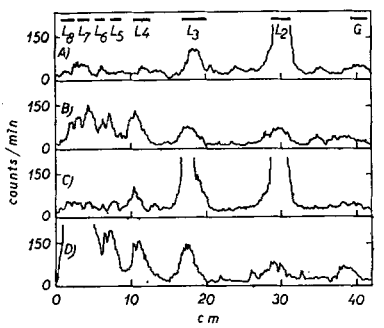


Fig. 5. Laminaribiose phosphorylase. Competition between laminaribiose and laminaritriose. (A) The incubation mixtures and enzyme activity were carried out as indicated in *Enzyme assay, Procedure B*, but containing in addition laminaritriose (●—●) as indicated. Laminaribiose phosphorylase originated from Peak I (Fig. 1) (0.007 units) was used to carry out these experiments. Under the same conditions, the K_m for laminaribiose and laminaritriose were 1 mM and 2.1 mM, and the $v_{max.}$ 4.6 and 1.2 mμmoles/min, respectively. (B) The conditions were similar to (A) but maintaining the amount of laminaritriose (0.5 μmole) constant and varying that of laminaribiose as indicated. Symbols as in Fig. 1. Solid line represents the theoretical curve (see text).

Fig. 6. β-1,3-oligoglucan phosphorylase. Competition between glucose and laminaribiose. (A) The incubation mixtures and enzyme were carried out as indicated in *Enzyme assay, Procedure A*, but containing glucose as indicated. β-1,3-oligoglucan phosphorylase originated from the rechromatography of Peak II (0.04 unit) was used to carry out these experiments. Under the same conditions, the K_m for glucose and laminaribiose were 45 and 4 mM, and the $v_{max.}$ 41 and 47 mμmoles/min, respectively. (B) The conditions were similar to (A) but with 40 mM glucose and varying the amount of laminaribiose as indicated. Solid line represents the theoretical curve. Symbols as in Fig. 1.

where a and b are the concentration of substrates, Va and Vb, their maximum velocities and K_a and K_b, their Michaelis constants. It can be seen that the experimental points obtained with both phosphorylases fall on the calculated curves, thus indicating the presence of only one active site in each enzymatic preparation.

Reaction products with glucose and laminaribiose as acceptors. It seemed interesting to compare the pattern of the reaction products obtained with both phosphorylases in view of their similar enzymic activities. For this purpose, laminaribiose- and β-1,3-oligoglucan phosphorylases were incubated separately with [^{14}C]Glc-1-*P* and glucose or laminaribiose as acceptor. The result of a radiochromatogram scanning after 15 and 45 min incubation is depicted in Figs. 7 and 8.



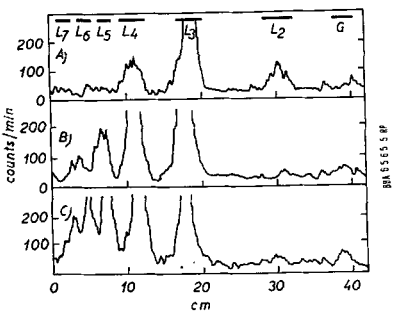


Fig. 7. Radioactive reaction products from both phosphorylases using glucose as acceptor. (A) Laminaribiose phosphorylase originated from Peak I (Fig. 1) was preincubated as described in text and afterwards incubated with 1 μmole of glucose and 0.6 μmole of [^{14}C]Glc-1-*P* (36 000 counts/min) for 15 min. (B) β-1,3-oligoglucan phosphorylase originated from the rechromatography of Peak II (Fig. 2) was preincubated as described in text and afterwards incubated as detailed in (A) during 15 min. (C) Similar to (A) but the incubation time was increased to 45 min. (D) Similar to (B) but the incubation time was increased to 45 min. The controls were carried out as above but omitting the acceptor. After stopping the reaction by heat, the content of the tubes was desalted, spotted on Whatman No. 1 paper and chromatographed in butanol–pyridine–water (6:4:3, w/v) for 20 h; the paper was dried and rechromatographed in the same solvent for another 20 h; this process was repeated once more. A scanning for radioactivity along the paper strip was carried out with a Nuclear Chicago Model D-47 gas flow counter fitted to a C-100A actigraph II with 0.5 inch collimator. G: glucose; L_2, laminaribiose; L_3, laminaritriose; L_4, laminaritetraose; L_5, laminaripentaose; L_6, laminarihexaose; L_7, laminariheptaose; L_8, laminarioctaose.

Fig. 8. Radioactive reaction products from both phosphorylases using laminaribiose as acceptor. (A) The conditions were similar to those described in Fig. 7C, but replacing 0.5 μmole of laminaribiose as acceptor. (B) Conditions as described in Fig. 7B but replacing 0.5 μmole of laminaribiose for glucose. (C) Conditions similar to (B) but after 45 min incubation. Other conditions and abbreviations as described in Fig. 7.

(a) Glucose as acceptor. Fig. 7A shows that laminaribiose phosphorylase formed laminaribiose and laminaritriose after short incubation periods; by increasing the time to 45 min, laminaritetraose could also be detected (Fig. 7C). The behavior of β-1,3-oligoglucan phosphorylase was, however, clearly different. Thus, as shown in Fig. 7B and D, a series of radioactive peaks ranging from laminaribiose to laminariheptaose were easily detected.

(b) Laminaribiose as acceptor. Laminaribiose phosphorylase formed laminaritriose, laminaritetraose and traces of laminaripentaose after 45 min incubation (Fig. 8A); the formation of some labeled laminaribiose was also observed, probably

due to the reversibility of the reaction. Shorter incubation periods were not run on account of the scarcity of the material produced as judged by the P_i released.

On the other hand, β-1,3-oligoglucan phosphorylase gave the same qualitative pattern as that observed when glucose was used as acceptor, except for the presence of labeled laminaribiose, after either 15 min or 45 min of incubation (Fig. 8B and C, respectively).

TABLE III

EFFECT OF MERCAPTOETHANOL, IODOACETATE AND *p*-MERCURIBENZOATE ON LAMINARIBIOSE AND β-1,3-OLIGOGLUCAN PHOSPHORYLASE ACTIVITY

A: The activity of laminaribiose phosphorylase was measured as follows: 2 μmoles of imidazole buffer, pH 7.2, 0.2 μmole of EDTA, additions in the concentrations indicated and 0.01 ml of enzyme originated from the first column chromatography (Fig. 1) in a final vol. of 0.03 ml. After preincubation at 30° during 20 min, 5 μmoles of glucose and 1 μmole of Glc-1-*P* were added. The mixture was incubated at 37° for 20 min and the P_i released was determined. Controls were run omitting the acceptor. B: In Expt. 1, the activity of β-1,3-oligoglucan phosphorylase was measured as above but using 0.01 ml of enzyme originated from the second column chromatography (1-week-old preparation; see Fig. 2) and replacing 0.5 μmole of laminaribiose or laminaritriose for glucose. In Expt. 2, the activity of β-1,3-oligoglucan phosphorylase was determined as described in Expt. 1 but with a 2-month-old enzyme and when indicated 5 μmoles of glucose were used. The incubation was performed for 10 min at 37°.

Addition	*Concentration (mM)*	*A (μmoles P_i released)*		*B (μmoles P_i released)*	
		Glucose	*Glucose*	*Laminaribiose*	*Laminaritriose*
Expt. 1					
None	—	0.36		0.19	0.17
Mercaptoethanol	20	0.36		0.48	0.42
Iodoacetate	0.2	0.37		0.15	0.12
Iodoacetate	2	0.31		0.04	0.03
p-Mercuribenzoate	0.007	0.37		0.06	0.03
p-Mercuribenzoate	0.07	0.04		0	0
Expt. 2					
None	—		0	0	0
Mercaptoethanol	20		0.20	0.24	0.24

Some experiments were carried out using laminaridextrins as acceptors for both enzymes, but the radioactive peaks found were not clearly separated from one another. However, a much lower degree of labeling could be observed with laminaribiose phosphorylase than with β-1,3-oligoglucan phosphorylase. In another experiment with the latter enzyme, a better separation among the radioactive peaks could be achieved and they were tentatively identified as laminarihexaose, laminariheptaose, laminarioctaose, laminarinonaose and laminaridecaose.

Finally, it must be pointed out that in all cases, controls were carried out similarly to the assays but omitting the acceptor. The scanning for radioactivity showed only a small peak in the zone corresponding to glucose, similar to that observed in Figs. 7 and 8. This was due to the presence of free [^{14}C]glucose in the [^{14}C]Glc-1-*P* solution, as was demonstrated by paper chromatography.

Comments

From the quantitative point of view, the results of β-1,3-oligoglucan phosphorylase activity using glucose as acceptor were rather puzzling when compared with those obtained with laminaribiose as substrate. It can be seen in Fig. 7B that, in the case of glucose, even after short periods of incubation, the higher oligosaccharides were more heavily labeled than the lower; this effect became more evident after longer incubations (Fig. 7D).

In the case of laminaribiose (Fig. 8B and D), as expected, a higher labeling was found in the lower homologues.

Further studies on the kinetic behavior of β-1,3-oligoglucan phosphorylase will be necessary to give a satisfactory explanation for this difference.

Miscellaneous experiments

As already stated, sulfhydryl groups seem to be essential for β-1,3-oligoglucan phosphorylase activity. This requirement was not easily detected in fresh enzyme preparations and was more evident in aged extracts. On the other hand, laminaribiose phosphorylase, in some experiments, was only slightly activated by mercaptoethanol.

The experiments described in Table IV were carried out in order to investigate the response of both enzymes to specific inhibitors of –SH groups. It can be seen that with glucose as acceptor, laminaribiose phosphorylase was only slightly inhibited by 2 mM iodoacetate, while a 90% inhibition was observed with 0.07 mM *p*-mercuribenzoate*. In the present assay, a higher dilution of the latter inhibitor was not effective. However in other experiments, a 50% inhibition was detected.

TABLE IV

COMPARISON OF DIFFERENT ACCEPTORS FOR β-1,3-OLIGOGLUCAN AND LAMINARIBIOSE PHOSPHORYLASES

In Expt. A, the enzymatic activity was measured as follows: 2 μmoles of imidazole buffer, pH 7.2, 0.2 μmole of EDTA, 1 μmole of mercaptoethanol (when added) and 0.01 ml of β-1,3-oligoglucan phosphorylase originated from the second chromatography of Peak II (see Fig. 2) (8 weeks old) were preincubated at 30° for 30 min; then 0.5 μmole of the acceptors listed below and 1 μmole of Glc-1-*P* were added and incubated at 37° for 20 and 40 min. The P_i released was determined as previously indicated. In Expt. B, the conditions were similar to Expt. A, except that 0.01 ml of laminaribiose phosphorylase originated from Peak I (Fig. 1) was used instead of β-1,3-oligoglucan phosphorylase. Controls were run omitting the acceptors.

Acceptor	*Incubation time (min)*	*A (μmoles P_i released)*		*B (μmoles P_i released)*	
		−SH	*+SH*	*−SH*	*+SH*
Laminaribiose	20	0	0.40	0.03	0.06
	40	0	0.59	0.05	0.10
Glucose	20	0	0.11	0.24	0.29
	40	0	0.28	0.45	0.47
α-Phenylglucoside	20	0	0.03	0	0
	40	0	0.08	0	0
α-Methylglucoside and laminarin	20	0	0	0	0
	40	0	0	0	0

* Several enzyme batches gave similar results with this concentration of *p*-mercuribenzoate. However it was about 30-fold lower than that previously reported[2] to obtain similar inhibition. These differences could not be explained.

Table IV also shows the results obtained with β-1,3-oligoglucan phosphorylase. A similar degree of inhibition was obtained with either laminaribiose or laminaritriose as acceptor using iodoacetate or *p*-mercuribenzoate as inhibitors. These results give additional evidences that the same active center is involved with the two oligosaccharides.

For comparison, the effect of a preincubation with mercaptoethanol on the activity of both enzymes is also given.

Expt. 2 demonstrates that aged preparations of β-1,3-oligoglucan phosphorylase were not active in absence of –SH donors using glucose, laminaribiose or laminaritriose as acceptors. Therefore, the results with glucose would support the suggestion that the enzyme preparation used in the experiment described in Fig. 3B was contaminated with some laminaribiose phosphorylase.

It might be appropriate to mention at this point that the activity of laminaribiose phosphorylase was not affected when assayed as described under *Enzyme assay, Procedure A* but substituting 20 mM hydroquinone for mercaptoethanol. On the contrary, the activity of β-1,3-oligoglucan phosphorylase was completely inhibited under the same conditions. As already mentioned[1], the latter effect was not observed when the enzyme activity was measured in presence of –SH donors.

MANNERS AND TAYLOR[4] reported that α-methyl- and α-phenylglucosides were poor acceptors for laminaribiose phosphorylase from *Astasia ocellata*, while laminarin was a good one. However, GOLDEMBERG, MARECHAL AND DE SOUZA[2] could not detect any activity with the mentioned substrates and the enzyme from *E. gracilis*.

As previously reported[1], some activity was found with α-phenylglucoside and β-1,3-oligoglucan phosphorylase. The assay was repeated but using both enzymes separated by DEAE-cellulose chromatography. Table IV shows that of the three mentioned substrates assayed only α-phenylglucoside was effective as acceptor and that β-1,3-oligoglucan phosphorylase was responsible for this activity. Although this result might explain the difference obtained with those given by MANNERS AND TAYLOR[4] for α-phenylglucoside, that corresponding to laminarin remains to be solved. In this sense, the presence of another phosphorylase (unpublished results) in crude extracts of *E. gracilis* which catalyzes the phosphorolysis of laminarin and KOH-treated paramylon, must not be discarded.

DISCUSSION

So far two β-1,3-oligoglucan phosphorylases have been found in cell-free extracts from *E. gracilis*: laminaribiose[2] and β-1,3-oligoglucan phosphorylases[1]. Both of them catalyze the same reaction but can be differentiated by several properties. One of the main characteristics is that β-1,3-oligoglucan phosphorylase shows a specific requirement for –SH donors as was demonstrated in the previous[1] and present papers. This is more marked with aged enzymatic preparations (see Tables III and IV). The kinetic behavior of the enzymes toward glucose or β-1,3-oligoglucans is also different. As shown in Table I, laminaribiose phosphorylase forms Glc-1-*P* from laminaribiose at a rate approx. 4-fold greater than with higher homologues, while the opposite was observed with the new enzyme.

Similar behavior was also detected when laminaribiose was used as acceptor for both enzymes. Fig. 3A shows that the reaction rate of β-1,3-oligoglucan phospho-

rylase was about 8-fold higher than laminaribiose phosphorylase at optimal concentrations of mercaptoethanol (see also Fig. 4). On the other hand, while laminaribiose phosphorylase retained 90–100% of its activity after 2–3 months[2], β-1,3-oligoglucan phosphorylase generally lost most of its catalytic effect in the same period (however, some exceptions were found, see Table IV).

As mentioned previously, one problem that remained to be solved was that related to the phosphorolytic action of laminaribiose phosphorylase on higher oligosaccharides and that corresponding to β-1,3-oligoglucan phosphorylase on laminaribiose. From the kinetic approach represented in Figs. 5 and 6, it can be concluded that the activities mentioned were due to properties of each enzyme and not to mutual contaminations (see also Table III, Expt. 2).

It seems interesting to point out that both phosphorylases, and especially β-1,3-oligoglucan phosphorylase, could form higher oligosaccharides starting with a molecule as small as glucose. As far as it is known, potato phosphorylase uses maltotriose or higher homologues as acceptors, while maltose is ineffective[5]. In the case of muscle phosphorylase, maltose appears to be the smallest glucosyl acceptor; however a long incubation time was necessary to detect this result[6]. On the other hand, other known disaccharide phosphorylases, sucrose and maltose and cellobiose phosphorylases, can act with fructose and glucose, respectively, as acceptors, but they only form the corresponding disaccharide. All these facts would differentiate the Euglena phosphorylase from that of other organisms. In this sense, it seems interesting that MANNERS AND TAYLOR[4] reported the presence of enzyme(s) that catalyze(s) similar reactions in extracts of Ochromonas and *Astasia ocellata* so that this type of enzyme seems ubiquitous in phytoflagellates.

Paramylon is considered to be the reserve carbohydrate of *E. gracilis* and other Euglenoids. BLUM AND BUETOW[7] demonstrated in *E. gracilis* var. bacillaris that most of the polysaccharide disappears after 13 days starvation, thus indicating the ability of the cells to utilize it as reserve carbon supply.

MERRIK AND DOUDOROFF[8] have found that a very complex mechanism is required to degrade the native poly-β-hydroxybutyric acid granules, the major reserve material of many types of bacteria. They also found that several physical or chemical agents make the grains resistant to digestion. These results could serve as a possible explanation for the fact that several attempts to obtain the *in vitro* breakdown of native paramylon grains with crude extracts of *E. gracilis* strain z were unsuccessful. As mentioned before, only a minute amount of Glc-1-*P* could be detected when these extracts were incubated with laminarin or KOH-treated paramylon.

At present, further studies seem to be necessary to elucidate the mechanism that governs the intracellular production of Glc-1-*P* from paramylon. However it is tempting to ascribe a physiological role to β-1,3-oligoglucan and laminaribiose phosphorylase. One can assume that the native paramylon granules yield oligosaccharides with a length of 8–10 glucosyl residues by some unknown mechanism, perhaps by the action of endoglucanases of the α-amylase type. This molecule is then degraded by β-1,3-oligoglucan phosphorylase giving Glc-1-*P* by phosphorolysis as well as an oligosaccharide with one less glucosyl residue. In this way we would get laminaribiose which would be further degraded by laminaribiose phosphorylase. This hypothetic mechanism is summarized in the following scheme:

$$\text{Paramylon} \xrightarrow{?} (\beta\text{-1,3-Glc})_n$$

$$(\beta\text{-1,3-Glc})_n + P_i \rightleftharpoons \alpha\text{-Glc-1-}P + (\beta\text{-1,3-Glc})_{n-1}$$
(β-1,3-oligoglucan phosphorylase)

$$(\beta\text{-1,3-Glc})_{n-1} + P_i \rightleftharpoons \alpha\text{-Glc-1-}P + \text{laminaribiose}$$
(β-1,3-oligoglucan phosphorylase)

$$\text{laminaribiose} + P_i \rightarrow \text{Glc} + \alpha\text{-Glc-1-}P$$
(laminaribiose phosphorylase)

There is some overlapping in the specificity of laminaribiose and β-1,3-oligoglucan phosphorylases towards the oligosaccharides. However, one might assume that the activity of β-1,3-oligoglucan phosphorylase on laminaribiose *in vivo* is insignificant as might be the case of laminaribiose phosphorylase towards the higher homologues, according to their respective *in vitro* reactions.

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