DEMONSTRATION AND PARTIAL PURIFICATION OF A ß-(1→3)-GLUCAN PHOSPHORYLASE

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Summary:

The present communication deals with the demonstration and tenfold purification of a phosphorylase from Ochromonas malhamensis acting on various algal reserve polysaccharides according to the equation:

$(ß\text{-}1\rightarrow 3\text{-glucan})_{n+1} + P_i \rightleftharpoons (ß\text{-}1\rightarrow 3\text{-glucan})_n +$ α-D-glucose-1-phosphate

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The phosphorolytic degradation of glycogen and starch is well studied with enzymes from animals and plants. Besides these α-(1→4)-glucans there is a second major group of reserve polysaccharides essentially characterized by ß-(1→3)-linked D-glucose chains and by the presence of a few ß-(1→6)-inter-chain linkages. Polysaccharides of this type constitute the main reserve carbohydrates of diatoms, golden brown and brown algae and are known under the trivial names of chrysolaminarin and laminarin[1,2,3]. These ß-(1→3)-glucans are substrates for the phosphorylase isolated from the golden brown flagellate Ochromonas malhamensis.

The enzyme was assayed during the procedure of partial purification measuring the formation of labeled polysaccharide from α-D-glucose-^{14}C-1-phosphate and laminarin or chrysolaminarin as an acceptor. Table 1 shows that the purification led to a tenfold increase in specific activity. The addition of protamine sulfate resulted in an apparent

Table 1. Purification of the β-1-3-glucan phosphorylase

Ochromonas was grown for 2 days in the light (7) and the cells from 2.5 l nutrient solution were centrifuged, washed with water and disrupted in 3 ml 0.05 M Tris-HCl, pH 8.6, with a Branson sonifier. The homogenate was centrifuged at 50 000 x g for 15 min. and the supernatant fractionated. The steps were: a) Addition of 3 mg protamine sulfate/ml. b) Addition of sat. ammonium sulfate, pH 7.3, 40 - 50 % saturation. c) Addition of 5 mg calcium phosphate gel per mg of protein and elution by 4 ml 0.05 M sodium pyrophosphate, pH 8.6. d) Adjusted to pH 4.3 with 10 % acetic acid. After dialysis for 2 h against 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, aliquot parts of the fractions were assayed for protein (9) and enzyme activity.
The incubation mixture for the enzyme assay contained 60 mM sodium acetate buffer pH 4.5, 0.6 mM EDTA, 5 mM sodium α-D-glucose-^{14}C-1-phosphate (0.43 mC/mmole), 0.25 mg laminarin and 3 to 50 µg of protein in a final volume of 0.05 ml. The mixture was incubated for 1 or 10 minutes, boiled, applied to a chromatogram and developed for 18 h in ethanol/0.5 M ammonium acetate pH 7.5, 1 mM EDTA = 60/40. The labeled polysaccharide remained at the starting line and was counted on the paper strip with 5 g PPO/l toluene in a Beckman LS 100 liquid scintillation system. One unit of enzyme transfers 1 µmole glucose per minute at 25°.

Fraction	Total Activity (Units)	Spec.Activity (U/mg protein)
Crude extract	5.3	0.14
Protamine sulfate, supern.	18.7	0.31
Ammonium sulfate prec.	11.0	0.45
Calcium phosphate gel eluate	7.1	0.50
pH 4.3, supern.	6.9	1.30

increase of total activity which may indicate that an inhibitor was removed by this step.

The polysaccharide formed was identified as follows: After paper-chromatography, it was solubilized fully from the paper by boiling it with 20 ml of water for 20 minutes. The labeled material was not retained on a column of Sephadex G 25 but came off the column with its exlusion volume indicating a molecular weight of about 4000 or higher. On partial hydrolysis (0.5 ml conc. HCl + 0.5 ml fuming HCl, 2 h, 25°) glucose, laminaribiose and a series of higher oligosaccharides were formed. The occurence of β-(1→3)-linkages was demonstrated by paper zone electrophoresis of the respective laminaribiitol in molybdate

Table 2. Acceptor specifity of the ß-1—3-glucan phosphorylase

The incubation mixture contained 55 mM sodium acetate buffer pH 4.5, 0.55 mM EDTA, 18 mM sodium α-D-glucose-1-phosphate, the indicated amount of acceptor and 16 µg of enzyme (similar to last line, table 1) in a total volume of 0.055 ml. After incubation for 20 minutes at 25° the reaction was terminated by boiling for 3 minutes and the inorganic phosphate liberated was determined (10). Soluble laminarin was purchased from C. Roth (Chem. Comp. Karlsruhe). Chrysolaminarin from Ochromonas was prepared by hot water extraction of dark grown cells. Acidic polysaccharides were precipitated from the extract with 0.05 % cetylpyridinium chloride and the chrysolaminarin recovered by precipitation with 10 volumes of ethanol. B.E. Volcani (La Jolla) kindly supplied a sample of chrysolaminarin from the diatom Navicula pelliculosa.

Acceptor present (source, mg)	endgroups (mM)	P_i liberated (µmoles/20 min.)	%
Water (control for phosphatase)	-	0.02	0
Laminarin (brown algae, 0.25)	2.2*	0.37	100
Chrysolaminarin (Ochromonas, 0.27)	2.2*	0.22	57
Chrysolaminarin (Navicula, 0.25)	2.2*	0.40	109
Cellobiose	2.2	0.10	29
Glucose	2.2	0.02	0
Starch, soluble (0.25)	-	0.02	0
Maltose	2.2	0.02	0
ß-Methylglucoside	2.2	0.02	0

* Nonreducing end groups were calculated on the basis of glucose content determined with glucose-6-phosphate-dehydrogenase (11) after hydrolysis (1 N HCl, 4 h, 100°) and roughly assuming a $\overline{DP}$ value of 30 and a $\overline{CL}$ value of 10 (2,3). This assumed concentration may differ from the actual concentration by a factor of about 2.

buffer[4]. This results indicate that the product was a polysaccharide to which labeled glucose residues were added in ß-(1→3)-linkage.

The specifity of the enzyme was determined by measuring the liberation of inorganic phosphate from glucose-1-phosphate in the presence of different acceptors (table 2). The highest activity was found with

Table 3. Time course and stoichiometry of the reaction

The reaction mixture contained 68 mM sodium acetate pH 4.5, 0.68 mM EDTA, 13.6 mM sodium α-D-glucose-^{14}C-1-phosphate (0.065 mC/mmole), 1.25 mg laminarin and 80 µg enzyme (similar to last line, table 1) in a final volume of 0.5 ml. Aliquots of 0.05 ml were removed and assayed for polysaccharide-^{14}C (same as table 1) and phosphate (same as table 2).

Incubation time (minutes)	P_i liberated (µmoles/0.05 ml)	ß-(1→3)-glucan formed (µmoles/0.05 ml)
10	0.09	0.094
20	0.185	0.190

ß-(1→3)-glucans, regardless of their origin from taxonomically different algae. Soluble starch cannot substitute for the ß-(1→3)-glucans. As the enzyme shows no activity with glucose or ß-methylglucoside it is definitely different from laminaribiose phosphorylase isolated from Euglena[5] or Astasia[6]. The fact that the addition of cellobiose led to the liberation of some inorganic phosphate may indicate that oligosaccharides containing ß-linked-glucose can also function as acceptors.

The validity of the equation given in the summary was demonstrated by the finding that the amount of phosphate liberated was equal to that of ß-(1→3)-glucan formed (table 3). The reversibility of the reaction is shown by the formation of α-D-glucose-1-phosphate from laminarin and inorganic phosphate (table 4). Although in this special experiment the reaction was not fully proportional to time, it clearly shows that the phosphorylase can degrade ß-1→3-glucans.

In analogy to the phosphorylases specific for starch and glycogen, the enzyme described in this communication may function in the metabolic mobilisation of reserve ß-(1→3)-glucans in algae. It may play an additional role in Ochromonas, as one step in the reversible trans-

Table 4. Formation of α-D-glucose-1-phosphate by the phosphorylase

The incubation mixture contained 70 mM potassium-sodium phosphate buffer, pH 6.5, 0.7 mM EDTA, 1.25 mg laminarin and 80 µg enzyme (similar to last line, table 1) in a final volume of 0.5 ml. The zero time control was withdrawn at 0° and the mixture transferred to 25°. Aliquots of 0.1 ml were boiled at the indicated time followed by determination of α-D-glucose-1-phosphate with phosphoglucomutase and glucose-6-phosphate-dehydrogenase (12).

Incubation time (min.)	0	10	30
α-D-glucose-1-phosphate formed (µmoles/0.1 ml)	0.02	0.145	0.285

formation of chrysolaminarin into α-galactosyl-(1→1)-glycerol which is of importance in regulating the osmotic balance of this flagellate[7,8].

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References:

1.) B.J.D. Meeuse, in: Biochemistry and physiology of algae, 289, Academic Press N.Y. (1962)
2.) A.R. Archibald, D.J. Manners and J.F. Ryley, Chem. and Industry 1516 (1958)
3.) M. Fleming and D.J. Manners, Biochem.J. 94, 17P (1965)
4.) E.J. Bourne, D.H. Hutson and H. Weigel, J.Chem.Soc. 4252 (1960)
5.) S.H. Goldemberg, L.R. Maréchal and B.C. De Souza, J.Biol.Chem. 241, 45 (1966)
6.) D.J. Manners and D.C. Taylor, Biochem.J. 94, 17P (1965)
7.) H. Kauss, Z.Pflanzenphysiol. 56, 453 (1967)
8.) H. Kauss, Nature, 214, 1129 (1967)
9.) O.H. Lowry, J.Y. Rosebrough, A.L. Farr, R.J. Randall, J.Biol. Chem. 193, 265 (1951)
10.) C.H. Fiske and Y. SubbaRow, J.Biol.Chem. 66, 375 (1925)
11.) M.W. Slein, in: Methoden der enzymat. Analyse, 117, Verlag Chemie Weinheim (1962)
12.) H.U. Bergmeyer and H. Klotsch, in: Methoden der enzymat. Analyse, 131, Verlag Chemie Weinheim (1962)