Phytochemistry, 1971, Vol. 10, pp. 1293 to 1298. Pergamon Press. Printed in England

PURIFICATION, CRYSTALLIZATION AND PROPERTIES OF A β-(1 → 3)-GLUCAN PHOSPHORYLASE FROM *OCHROMONAS MALHAMENSIS*

G. J. ALBRECHT* and H. KAUSS†

Institut für Botanik der Technischen Hochschule Munchen, D 8000 Munich 2, Arcisstr. 21, Germany

(*Received* 20 *July* 1970)

Abstract—A method for the crystallization of a β-(1 → 3)-glucan phosphorylase (laminarin phosphorylase) from the golden brown alga *Ochromonas malmensis* is described. The enzyme is optimally active at pH 5 5 and at a temperature of 22 5°. The apparent K_M-values determined for laminarin, inorganic phosphate and glucose-1-phosphate were about 10^{-3} M, $2{\cdot}5 \times 10^{-2}$ M and $1\,2 \times 10^{-2}$ M, respectively. The enzyme is stimulated by low concentrations of AMP and inhibited by high concentrations of AMP. The apparent K_M-value for this activator was 4×10^{-5} M and its apparent K_I-value 2×10^{-2} M.

INTRODUCTION

ENZYMES, which catalyse the phosphorolysis of oligosaccharides with β-(1 → 3)-glucosidic linkages have been described from various sources.[1–3] More recently, Kauss and Kriebitzsch[4] demonstrated that *Ochromonas malhamensis* contains a similar enzyme which, however, can act on polysaccharides of the laminarin type. Such β-(1 → 3)-glucans constitute the main reserve polysaccharides of diatoms, golden-brown, and brown algae.[5–7] The purification, crystallization, and some properties of this phosphorylase are reported in this paper.

RESULTS

As seen from Table 1, an overall purification of about 50-fold from the crude extract was achieved by the usual methods (Fig. 1). Although the yield was low, the procedure resulted in a crystallization of the enzyme as fine needles (Fig. 2). Assuming the crystalline enzyme protein to be fairly pure with a specific activity of about 10 units per mg protein the enzyme would constitute about 2–2·5% of the extractable protein in the crude extract.

Polyacrylamide gel electrophoresis of the crystalline enzyme protein showed one faint band and a broad band. Visually, from the intensity of the stained bands and from the width of the bands, a purity of about 80% or more was estimated.

* Present address Pharmakologisches Institut der Universität München, 8000 München 15, Nußbaumstr. 26, Germany.

† Present address Botanisches Institut der Universität München, 8000 München 19, Menzingerstr. 67, Germany.

[1] S H. GOLDEMBERG, L R MARÉCHAL and B. C DESOUZA, *J. Biol. Chem.* **241**, 45 (1966)
[2] L R. MARÉCHAL and S H GOLDEMBERG, *Biochem Biophys. Res. Comm* **13**, 106 (1963).
[3] D. J. MANNERS and D C. TAYLOR, *Biochem J* **94**, 17 P (1965)
[4] H KAUSS and C. KRIEBITZSCH, *Biochem Biophys Res. Comm.* **35**, 926 (1969).
[5] B. J D. MEEUSE, in *Biochemistry and Physiology of Algae*, p 289, Academic Press, New York (1962).
[6] A R ARCHIBALD, D. J. MANNERS and J F RYLEY, *Chem & Ind* 1516 (1958)
[7] M FLEMING and D J. MANNERS, *Biochem J* **94**, 17 P (1965).

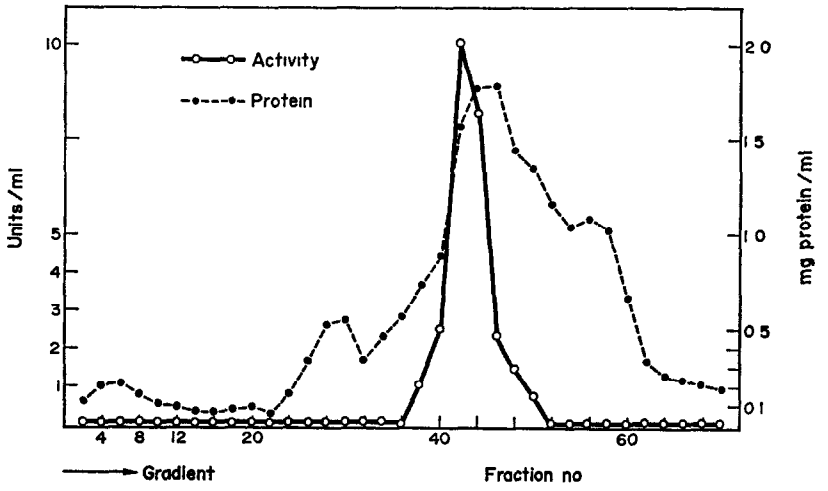

FIG. 1. ELUTION PATTERN FROM DEAE CELLULOSE CHROMATOGRAPHY.
The elution pattern of the protein from the DEAE column was done with a NaCl gradient as described in the text. Protein was measured with the method by Warburg and Christian[14] and the activity was assayed with assay A in each other fraction.

TABLE 1. PURIFICATION OF THE β-(1 → 3)-GLUCAN PHOSPHORYLASE: THE ENZYME ACTIVITY WAS TESTED WITH ASSAY A WITHOUT ADDITION OF AMP

Step	Vol. (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Purification-fold
1 Crude	570	8 0	4560	1 71	975	0 21	(1)
2. Protamine sulphate	600	6 5	3900	3 00	1800	0 46	2·16
3 $(NH_4)_2SO_4$	72	11 0	792	25 5	1856	2 32	10 8
4. Acid step	70	5 0	350	25 5	1785	5 10	23 8
5. DEAE cellulose	50	1 6	80	10 2	510	6 38	29·8
6. $(NH_4)_2SO_4$	6 3	2 2	13 8	22 8	143 6	10 35	48·5
7. First crystals	1 0	7 5	7 5	82 0	82 0	10·92	51 0
8. Second crystals	1 0	5 5	5 5	60 0	60 0	10 92	51 0

Some properties of the phosphorylase preparation are demonstrated by the experiment reported in Fig. 3. After the addition of laminarin the reaction rate is linear for at least 5 min. Moreover, the curve shows that the enzyme requires a primer, in this case laminarin, and that glucose-1-phosphate alone cannot act as a primer to start the reaction. Figure 3 also shows (preincubation time) that the enzyme preparation is free of any glucose-1-phosphate phosphatase which might have disturbed the assay, thus ensuring the enzymic reaction of laminarin phosphorylase. In similar experiments it was found that the rate of enzyme reaction is proportional to the amount of enzyme protein.

The reaction rate departs from linearity after about 5–7 min (Fig. 3), the exact time depending on the concentration of enzyme used. This is difficult to explain since product inhibition or a rapid decrease in substrate concentration can be excluded because the K_M-values are in the millimolar region and only a few micromoles are measured in the test

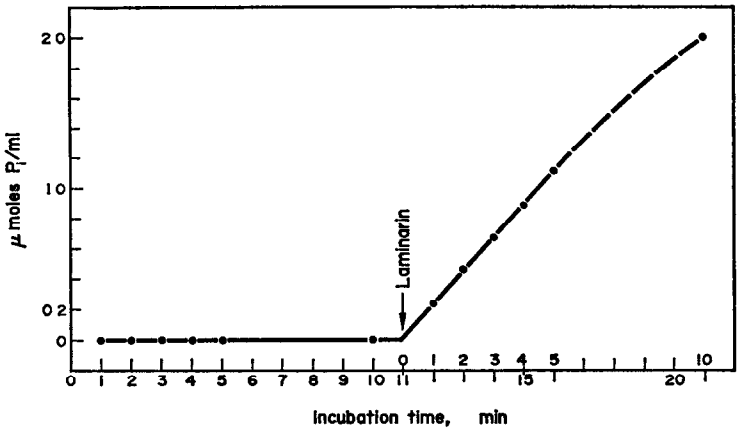

FIG. 3. ENZYMIC ACTIVITY AS A FUNCTION OF TIME AND ACCEPTOR.

Standard assay A conditions were used, incubation with enzyme in a total volume of 1·5 ml removing appropriate amounts of test mixture at the indicated time intervals. After 10 min the reaction was started with laminarin as acceptor and again 100 µl of reaction mixture each were removed.

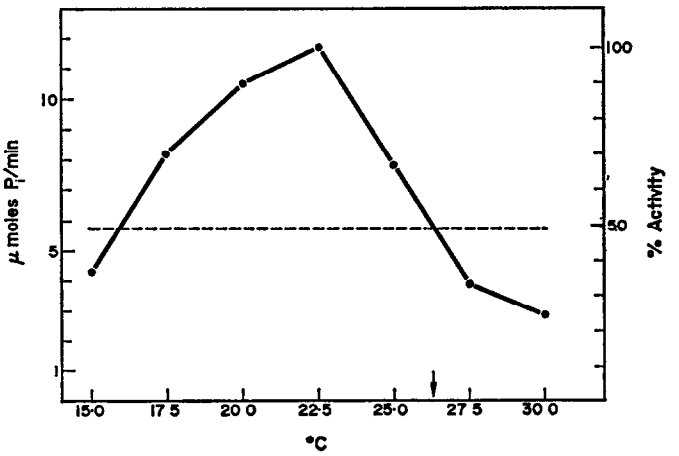

FIG 4. TEMPERATURE OPTIMUM OF THE ENZYME.

The data were obtained with assay A by incubating both the test mixture and the enzyme solution separately at the desired temperature for at least 10 min or longer before the reaction was started by adding a constant amount of enzyme to the individual tubes.

assay. The enzyme seems to be specific for glucose-1-phosphate, since glucose-6-phosphate, glucose-1,6-diphosphate, fructose-1-phosphate, fructose-1,6-diphosphate (all in a concentration of $2{\cdot}5 \times 10^{-2}$ M), could not replace glucose-1-phosphate as substrate.

Both for laminarin synthesis and phosphorolysis, a pH of 5·5 was found to be optimal, 50% of the maximum activity being achieved at pH 5·0. The activity increases rapidly up to pH 5·5, and decreases slowly with higher pH values. The enzyme seemed to be stable over several weeks even in crude extracts when kept between 0–4°. Storage at −20°, freezing and thawing resulted in a greater loss of activity.

As seen from Fig. 4, optimum enzyme activity is reached at 22·5° and it decreases rapidly with increasing temperature. Less than 50% of the maximum activity is reached at 27°. This unusual temperature sensitivity is difficult to interpret; it does not appear to represent an effect of the temperature on the stability of the enzyme. This was confirmed in the following way. After the experiments were run, the same enzyme solution, which had been incubated at the stated temperature for about 1 hr, was used for a second experiment in which the same activity was observed as before. This means that the enzyme was obviously not damaged during the incubation time. Following this finding, all experiments were run at 22·5°.

The affinities of the glucan phosphorylase towards its substrates laminarin, inorganic phosphate, and glucose-1-phosphate were measured using Lineweaver–Burk plots[8] and were 1·6 g/l, $2{\cdot}5 \times 10^{-2}$ M and $1{\cdot}2 \times 10^{-2}$ M, respectively. From the observed K_M-value of 1·6 g/l for laminarin the apparent K_M-value in respect ot the endgroups can be calculated to be about 10^{-3} M or slightly lower.[4] The affinity of the phosphorylase, therefore, seems to be greater for the polysaccharide than for glucose-1-phosphate. As the concentration of glucose-1-phosphate in the cells might be very low, this would favour degradation of polysaccharide. The enzyme would, therefore, mainly function in the mobilization of the reserve polysaccharide. As the K_M-values for the three substrates seemed to be rather high, an unknown factor appeared to be missing from the enzyme preparation. However, Mg^{2+} (in a concentration up to 10^{-2} M), EDTA (up to 2×10^{-3}M), alone or in combination with each other, cysteine, vitamin B_6, and pyridoxal phosphate (up to 5×10^{-6} M) did not stimulate or inhibit enzyme activity.

A considerable increase of enzyme activity was observed when the incubations were carried out with increasing amounts of AMP (Fig. 5). The effect is optimal at a concentration of about 10^{-3} M AMP and decreases significantly at higher AMP concentrations. As seen from Fig. 5 a 2,1-fold increase of the activity can be achieved with the proper amount

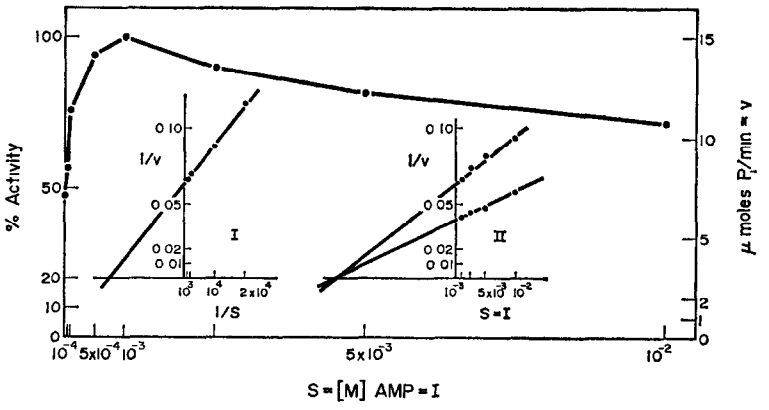

FIG. 5. INFLUENCE OF AMP ON THE PHOSPHORYLASE REACTION.

To the incubation mixture of assay A increasing amounts of AMP as indicated were added. The curve allowed to plot the data according to Lineweaver and Burk[8] to determine the apparent K_M-value (insert I) and apparent K_I-value (insert II) for AMP The two curves in insert II show a competitive type of inhibition as derived from two different glucose-1-phosphate levels in the experiment: upper line represents changing AMP concentrations as indicated at a concentration of 0·0125 M G-1-P; the lower line resulted from a 0·025 M G-1-P concentration at the indicated AMP levels.

[8] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

of AMP in the incubation mixture. The insert I in Fig. 5 allows the apparent K_M-value for AMP to be estimated as 4×10^{-5} M using the data where the concentrations of AMP were less than 10^{-3}M. For AMP concentrations higher than 10^{-3}M, two experiments at different substrate levels (glucose-1-phosphate) are included in insert II of Fig. 5. They indicate that the inhibition is of a non-competitive type.

A 2,2-fold increase of the enzyme activity could be observed when enough AMP (10^{-3} M) was added to the incubation mixture at optimal standard conditions (see also Fig. 5). However, we could not find any effect of this activator on the apparent K_M-values of the phosphorylase in regard to its substrates laminarin, inorganic phosphate and glucose-1-phosphate.

All the experiments except Fig. 5 were performed without the addition of AMP. Fig. 5 shows that about half of the activity of the enzyme preparation can be measured without addition of the activator. This could be explained by the assumption that the phosphorylase even after the various purification steps still contains some AMP derived from the cells. Another possible explanation would be that part of the phosphorylase molecules are not subject to the activation by AMP but exist already in the cells in a form active without AMP. A similar case is well known for glycogen phosphorylase from muscle where one form is active only with AMP and the other form active without AMP.[9] The elucidation of such regulatory aspects for the β-(1 → 3)-glucan phosphorylase must await further experiments. Some preliminary data show that the activity of the enzyme is possibly influenced also by ATP, ADP and 3′-5′-cyclic AMP.

EXPERIMENTAL

Ochromonas was grown in the light in 10 l. bottles under aeration and at room temp. (20–23°) similar to the method employed by Kauss.[10] After 3–5 days the cells were harvested by centrifugation, washed once with cold water, and stored in a thick suspension at −20° in 0·05 M Tris-HAc buffer, pH 7·0.

Assay A. The mixture contained in a final volume of 0·2 ml. 0·5 M Na-citrate buffer, pH 5·5, 0·025 M glucose-1-phosphate, 3 mg/ml laminarin, and sufficient enzyme to yield about 0·01–0·08 units. Incubation was for 0–4 min at 22·5°. The reaction was terminated by addition of 0·05 ml 50% (w:v) TCA. The inorganic phosphate liberated was determined by the method by Fiske and Subbarow.[11]

Assay B. The mixture contained in a final volume of 0·2 ml· 0·5 M Na-citrate buffer, pH 5·5, 0·05 M inorganic phosphate, 3 mg /ml laminarin, and sufficient enzyme. After 0–4 min incubation at 22·5° the reaction was stopped by heating the sample to 100° for 2 min. The liberated glucose-1-phosphate was measured in an aliquot by converting it to glucose-6-phosphate with phosphoglucomutase (E.C. 2.7.5.1) and subsequently to 6-phospho-gluconate with glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and NADP according to [12] One unit of enzyme is defined as that amount which liberates 1 μmole of product per min at 22·5°.

Protein determination. The protein content was determined either by a modified Biuret method[13] or by measuring at 280/260 nm [14]

Purification Procedure

Unless otherwise stated, all steps were carried out at 0–4°, all centrifugations were at 48,000 *g* (0°), and buffers used throughout the purification procedure were 0·05 M Tris-HAc, pH 7·0.

1. *Extraction of the enzyme.* The thawed algal cells were disrupted by sonification with a BRANSON sonifier for 15 sec (×4) and 1 ml of diisopropylfluorphosphate (DFP) was added to 1 l. of sonified algae. Cell debris was removed by centrifugation for 15 min. The supernatant was fraction I.

[9] H. Holzer, *Advan Enzymol* **32**, 297 (1969).
[10] H Kauss, *Z. Pflanzenphysiol* **56**, 453 (1967)
[11] C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375 (1925).
[12] A. Munch-Petersen, *Acta Chem Scand.* **9**, 1523 (1955)
[13] A. Kröger and M Klingenberg, *Biochem Z* **344**, 317 (1966).
[14] O. Warburg and W. Christian, *Biochem. Z* **310**, 384 (1941).

2. *Protamine sulfate.* To fraction I 0·2 vol. of 3% protamine sulfate was added at pH 6 9 and stirred for 5 min The supernatant from a centrifugation for 10 min was fraction II. Usually an increase of the enzymic activity (about 2-fold) was observed [4]

3. *Ammonium sulfate.* Fraction II was brought to 33% saturation by addition of $(NH_4)_2SO_4$[15] at pH 6·8 and stirred for about 30 min. After centrifugation more salt was added to the supernatant to bring the saturation to 0 5. The precipitate was taken up in a smallest possible volume of buffer and dialysed against two changes of buffer overnight (fraction III).

4. *Acid treatment.* Fraction III was brought to pH 4 3 with concentrated acetic acid and stirred for 5 min. The resulting precipitate was discarded. The supernatant, re-adjusted to pH 7·0, was fraction IV.

5. *DEAE cellulose chromatography.* Fraction IV was placed onto a DEAE-column (2 × 50 cm) and washed with about 1 l. of buffer. Then a linear gradient was applied: 250 ml of buffer in a mixing flask and 250 ml of buffer containing 0 25 M NaCl in an overhead container. Fractions of about 7 ml were collected and assayed for activity and protein. The active fractions were pooled, yielding fraction V.

6. *Concentration with ammonium sulfate.* Fraction V was brought to 50% $(NH_4)_2SO_4$ concentration and the pellet extracted 3 times with *ca.* 2 ml of cold water. The pooled supernatant constituted fraction VI.

7. *Crystallization.* Fraction VI was allowed to stand at room temp overnight. The crystals which formed were collected by centrifugation for 15 min at 5000 rev/min at room temp. and taken up in *ca.* 1 ml ice-cold water. Denatured protein was removed by centrifugation in the cold. The clear supernatant was fraction VII.

8. *Recrystallization.* For recrystallizing, the enzyme fraction VII was treated as before.

[15] L. NODA and S. A KUBY, *J. Biol. Chem.* **266,** 541 (1957).