Convenient Detection of β-1, 3-glucan Phosphorylases of Euglena gracilis z by Polyacrylamide Disc Gel Electrophoresis

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Convenient Detection of β-1, 3-glucan Phosphorylases of *Euglena gracilis* z by Polyacrylamide Disc Gel Electrophoresis

Kazutaka MIYATAKE and Shozaburo KITAOKA

Nutrition Laboratory, College of Agriculture

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Abstract

Using polyacrylamide disc gel electrophresis, a simple and convenient staining method for β-glucan phosphorylase was developed. With this method the phosphorylase could be demonstrated as sharp bands and from the electrophoretic mobility, *Euglena gracilis* phosphorylases could be classified into two: laminaribiose phosphorylase (glucose specific) and the β-1, 3-oligoglucan phosphorylase (laminaribiose or larger β-1, 3-oligomer specific).

Introduction

Several reports described the presence of isozymes of α-glucan phosphorylase, using polyacrylamide disc gel electrophoresis.[1-5] To demonstrate phosphorylase activity, two activity staining techniques were used. The first method was based on Gomori's technique, in which inorganic phosphate liberated from glucose-1-phosphate was converted to lead phosphate, which was then detected as lead sulfate.[1-3] The second one was based on the iodine glycogen color reaction, in which glycogen formed from glucose-1-phosphate was detected by iodine.[4,5]

β-glucans are not stained by iodine, and so we improved the first method.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane, glucose-1-phosphate, acrylamide, N,N, N′,N′-tetra methylethylenediamine (TEMED), N,N′-methylenebisacrylamide(BIS), bromophenol blue (BPB) and Coomassie brilliant blue were purchased from Nakarai Chemicals. All chemicals were analytical grade reagents prepared in Japan.

Assay method. Phosphorylase activity in cell free extract of *E. gracilis* was assayed according to Maréchal[6]. Protein was determined by the method of Lowry et al[7].

Preparation of extract of E. gracilis. E. gracilis, strain z, was stock-cultured in the Hutner's medium which contained (g/l) 1.12 $(NH_4)_2HPO_4$, 0.5 $MgSO_4$, 0.2 $CaCO_3$, 10 glucose and small amounts of minor salts and vitamins B_1 and B_{12} ; the initial pH was 3.3[8]. Cultivation was conducted in the dark at 27°C with aeration. *E. gracilis* cells were harvested, suspended in 50 mM acetate-sodium buffer, pH 7.0, and disrupted by sonication. The sonicated solution was centrifuged at 20,000 × *g* for 15 min and the supernatant was used as the crude enzyme.

Disc gel electrophoresis. Polyacrylamide gel disc electrophoresis was carried out by the procedure of Ornstein and Davis[9]. Gel tubes 7.5 cm long 0.5 cm in diameter were used. A 0.05–0.1 ml of quantity of the sample solution (in 50% sucrose solution) of

phosphorylase was applied between the running buffer solution and the spacer gel. Electrophoresis was carried out 75V, and 1 mA per tube at 4°C, until tracking dye band (BPB) moved about 5.0 cm through the separating gel. Protein fractions were stained by 0.5% Coomassie brilliant blue in 7% acetic acid and phosphorylase activity was visualized by staining by the following method.

Staining phosphorylase activity in acrylamide gel. The method developed for staining phosphorylase activity in a polyacrylamide gel is a modification of the Gomori's histochemical procedure as adapted for staining phosphatase activity in gel matrixes[10]. Following electrophoresis, the gels were incubated at 37°C for 30 min in a substrate solution at pH 7.0 containing 0.03 M glucose-1-phosphate, 0.2 M $CaCl_2$, 40 mM glucose or 5 mM laminaribiose, and 0.05 M sodium maleate in the presence or absence of 0.01 M 2-mercaptoethanol. The gels were washed briefly with deionized water at room temperature and were then placed in a solution containing 80 mM Tris-maleate and 3 mM $Pb(NO_3)_2$ at pH 7.0. After one hour at 25°C, the gels were again washed with several changes of deionized water over a period 1 to 12 hours and were placed for 5 min in a solution containing 5% ammonium sulfide. Washing with water was repeated. The developed gels were stored in the dark in water in stoppered tubes. Densitometric tracings were performed by scanning with a densicord recording electrophoresis densitometer, model FD-AIV (Fuji-Riken) with a gel carriage adaptor; six filters were used.

Results and Discussions

Detection of phosphorylase in cell free extract of ***Euglena*** *by Polyacrylamide gel staining.*

In Fig.1 Coomassie brilliant blue staining of an *Euglena gracilis* extract (A) and its phosphorylase activity in the presence (B) and absence (C) of glucose in the incubation

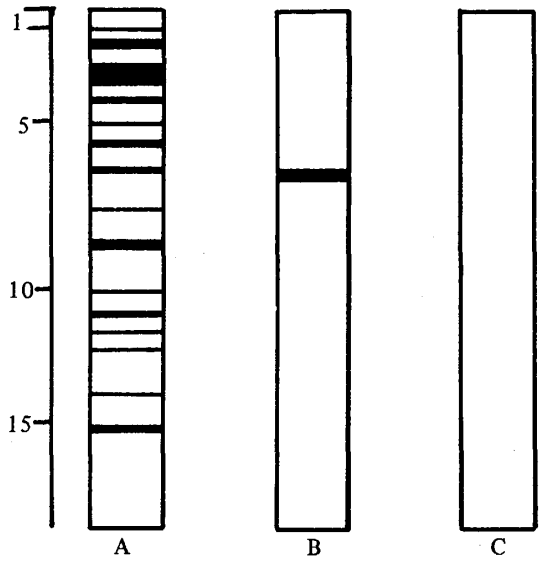

Fig. 1. Polyacrylamide gel electrophoresis of *E. gracilis* cell free extract.
A. Protein fractions with Coomassie brilliant blue.
B. Phosphorylase activity staining after incubation in the presence of glucose.
C. Phosphorylase activity in the absence of glucose.

solution are shown. The protein fractions were separated into about 12-15 bands. One phosphorylase activity band appeared in activity staining in the presence of glucose but not in its absence. When the protein bands were numbered from the cathode to the anode of the gel, the phosphorylase band corresponded to the 7th protein band.

Absorption spectrum of stained gel.

Densitometric tracings were carried out by scanning with a densicord recording electrophoresis densitometer with several filters (420, 450, 500, 530, 570, 600 nm). As shown in Fig.2 stained gel gave the most intensive absorption at 450-500 nm (a) and optical density at 450nm increased in a linear relation with enzyme concentration (b).

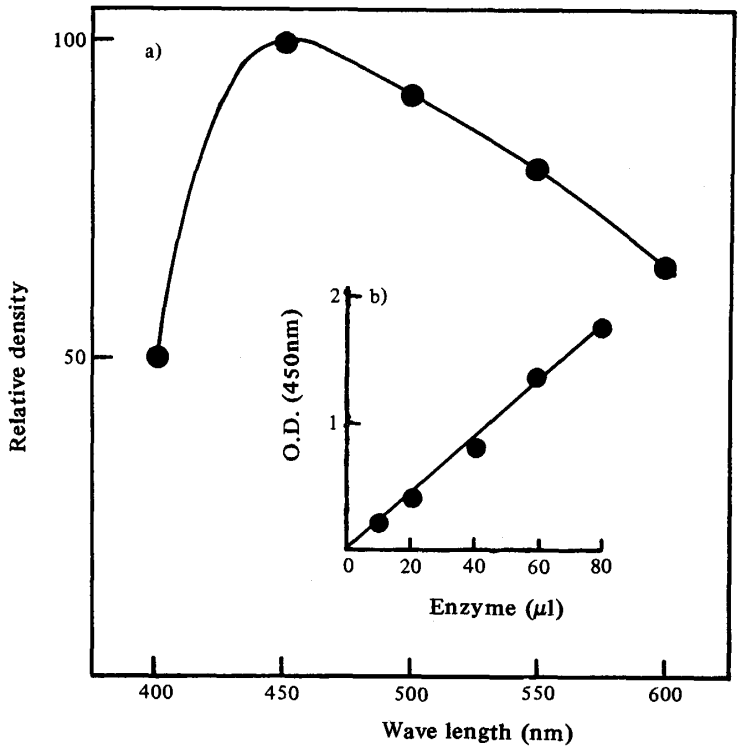

Fig. 2. Absorption spectrum of stained gel at several wave langhts.
a) Absorption spectrum were made by scanning with a densicord recording electrophoresis densitometer.
b) The amount of phosphorylase in the gel.

Substrate specificity of Phosphorylase.

To study the specificity of *Euglena* phosphorylase, some mono- and disaccharides were tested. Fig 3. shows that glucose is the best acceptor for glucose-1-phosphate and laminaribiose is less efficient. Other mono- (galactose, 2-deoxyglucose, mannose, fructose and glucosamine) and other disaccharides (cellobiose, sucrose, trehalose, gentiobiose, lactose and maltose) were not the substrates for *Euglena* phosphorylase by the present staining assay method.

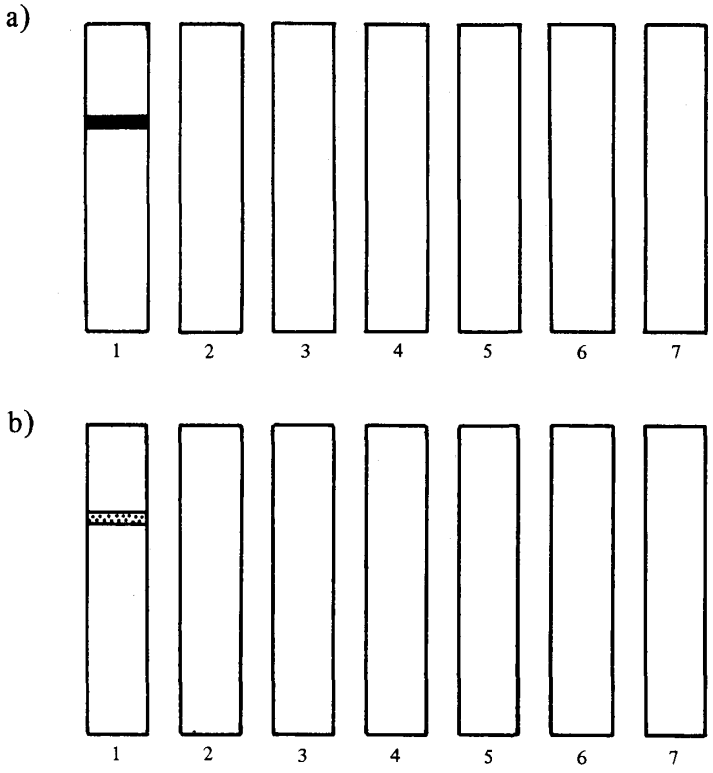

Fig. 3. Disc gel electrophoresis of *E. gracilis* phosphorylase with several acceptors.
Acceptors; a) Monosaccharides; 1: glucose, 2: fructose, 3: mannose, 4: galactose, 5: 2-deoxyglucose, 6: glusosamine, 7: sorbitol
b) Disaccharides; 1: laminaribiose, 2: cellobiose, 3: sucrose, 4: gentiobiose, 5: maltose, 6: lactose, 7: trehalose.
Each acceptor is contained in 0.1% in the separation gel.
■ : Deep color development
▨ : Pale color development

Effect of mercaptoethanol on phosphorylases.

A sole activity band was found in the polyacrylamide gel when laminaribiose was used as the substrate of the enzyme in the absence of SH reagent (A). Phosphate was liberated from glucose-1-phosphate when glucose was a substrate in the absence of SH reagent (B) while two activity bands appeared in the presence of 2-mercaptoethanol when laminaribiose was used as a substrate (C). The protein fractions were separated into 12-15 bands from cathode to anode of the gel. Phosphorylase activities in the gel corresponded to the protein bands Nos. 5 and 7, in the presence of 2-mercaptoethanol when laminaribiose was used as a substrate (C). The results show the presence of two phosphorylases in *Euglena,* one is glucose specific and SH independent and other is laminaribiose specific and requiring an SH reagent for phosphorylase activity.

Occurrence of two types of phosphorylase in *Euglena gracilis* have been reported by Maréchal [11]. He reported that both phosphorylases were separated by column chromatography and the two enzymes differed in their requirement for compounds with SH-group; the effect was especially prominent in reaction mixture containing laminaribiose as a acceptor[11].

The present result on the effect of SH reagent on phosphorylase was in fair accor-

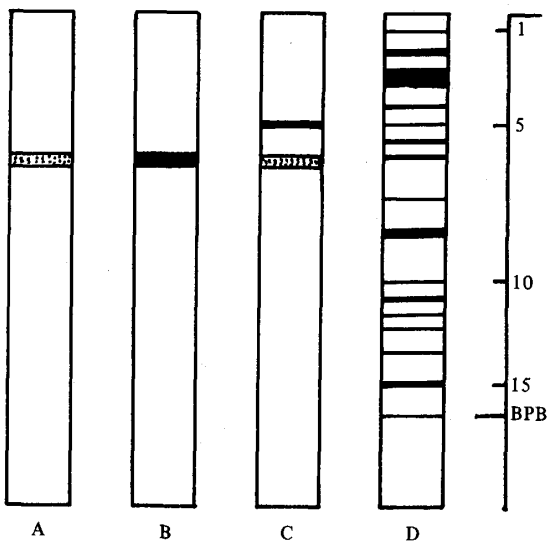

Fig. 4. Activity patterns of separated *Euglena* phosphorylases on the disc gel electrophoresis.
A : With laminaribiose (5 mM) as substrate without SH reagent.
B : With glucose (40 mM) as substrate without SH reagent.
C : With laminaribiose (5 mM) as substrate with SH reagent (10 mM).
D : Protein fractions as stained with Coomassie brilliant blue.
BPB ; bromophenol blue
; Deep color development
; Pale color development

dance with the reported one[11].

Many organisms containing a β-1, 3-glucan are believed to have β-1, 3-glucan phosphorylases in their cells, but a convenient method for its detection has not been known. The present gel staining method is easily applied for detection of phosphorylases and also for clarifying the specificity of the enzyme.

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