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Short Communication

Fast and sensitive simultaneous staining method of Q-enzyme, α-amylase, R-enzyme, phosphorylase and soluble starch synthase separated by starch–polyacrylamide gel electrophoresis

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ABSTRACT

The present paper describes fast and sensitive techniques for simultaneous activity staining of Q-enzyme, α-amylase, R-enzyme, phosphorylase and soluble starch synthase on starch–polyacrylamide gel. Staining of Q-enzyme on starch–polyacrylamide gel electrophoresis (PAGE) leads to red bands on the blue background. The α-(1→6) transferred short-cahins cause a shift of λ_{max} of the iodine–starch complex to shorter wavelengths. R-enzyme is visible as a light-blue band, whereas the α-(1→4) hydrolytic activity of α-amylase causes a colourless band and can be clearly distinguished from Q-enzyme. White bands of phosphorylase and soluble starch synthase are visualized and the substrate specificity of phosphorylase is discussed, since the enzyme incorporates α-D-glucosyl residues in the growing α-(1→4) chain from ADP-glucose or its decomposition product as a substrate. The presented staining techniques permit the activity staining of the most important enzymes of the starch biosynthesis and offer simple methods of detection on starch–PAGE.

INTRODUCTION

Branching enzyme [Q-enzyme, (1→4)-α-D-glucan: (1→4)-α-D-glucan 6-glucosyltransferase, EC 2.4.1.18] was first detected in potato juice [1] and has been isolated during the years from various plants [2–13], animal tissue [14,15] and bacteria [16,17]) where starch is found as a storage polysaccharide. The action of Q-enzyme can be followed by the increasing number of (1→6) linkages, shown by the increased resistance towards β-amylolysis [18].

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Many authors have suggested that the synthesis of starch or glycogen is based on by the cooperation of Q-enzyme and phosphorylase [(1→4) α-D-glucan-orthophosphate-glucosyltransferase, EC 2.4.1.1] [19–21] or starch synthase [(ADP-glucose: (1→4)-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.21] [22–25]. Usually the enzymic action *in vitro* is measured via inorganic phosphate, delivered from glucose 1-phosphate by phosphorylase or photometrically by the iodine staining method. This method, although rather unspecific and susceptible to interference in the presence of α-(1→4)-hydrolyzing enzymes, is unfortunately used for kinetic studies of Q-enzyme [26]. An activity staining method was performed by incubating the polyacrylamide gels

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after electrophoresis in an amylose solution [27] to observe the changes in λ_{max} of the iodine stain which is shifted to shorter wavelengths by the action of Q-enzyme. In a reaction based on the same principle phosphorylase is entrapped within the running gels [28] to synthesize glycogen within the gel as a substrate for subsequent activity staining of the branching enzyme. The described staining methods are suitable for multiple enzyme detection in crude or purified extracts. Activity detection of Q-enzyme, α-amylase, R-enzyme, phosphorylase and soluble starch synthase is shown on the same gel plate from starch–PAGE.

EXPERIMENTAL

Chemicals

Potato starch for electrophoresis (S-4501), glucose 1-phosphate, ADP-glucose and Coomassie Brilliant Blue R-250 (B-0149) were purchased from Sigma (Munich, Germany). All other chemicals were of analytical grade (Merck, Darmstadt, Germany). Pullulanase EN-200 (40 U/mg) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan).

Preparation of enzyme extract

Young potatoes were treated as described previously [29] and immediately blended with 10 ml of 1 *M* tris–citrate buffer (pH 7.5), containing 2.5 m*M* 1,4-dithio-DL-threitol.

Determination of protein content

After centrifugation protein determination with Lowry reagent was performed spectrophotometrically at 690 nm [30].

Starch polyacrylamide gel electrophoresis (starch–PAGE)

Gel electrophoresis was performed in a vertical slab gel apparatus. A separation gel with 15.0 % acrylamide, (pH 8.6), crosslinked 1:75 with N,N′-methylenebisacrylamide containing 1% starch was used. Starch synthase and phosphorylase (Fig. 2) were separated on 7.5 % acrylamide, (pH 8.6), crosslinked 1:75 with N,N′-methylenebisacrylamide containing 1% starch. 50 μl of the potato extract were sufficient for band detection. For detection of R-enzyme activity, which was negligible in the potato juice 5 μl of pullulanase were added. The electrophoresis buffer system consisted of tris–glycine (pH 8.9). The separation time was three h with a constant voltage of 300 V and a starting current of 120 mA. After electrophoresis the gels were rinsed in distilled water for 20 min.

Detection of Q-enzyme, R-enzyme and α-amylase activity

After electrophoretic separation the gel was soaked in 50 m*M* tris–citrate buffer (pH 7.0) containing 2 m*M* ascorbic acid for 4 h at 22°C. Afterwards the gel stripes were rinsed with distilled water and incubated in an iodine solution according to ref. 29 for 15 min. The zones of Q-enzyme activity appeared as sharp red bands on the blue stained background. R-enzyme activity could be seen as a light blue band, whereas α-amylase showed clear and transparent bands on the blue background. Staining of reference stripes with Coomassie Brilliant Blue R-250 [0.25% in methanol–glacial acid–water 5:1:5)] permits detection of the protein pattern matching to the activity stain.

Detection of soluble starch synthase activity (ADP-glucose)

Gel stripes incubated in a test tube containing 100 m*M* NaOH–glycine buffer, (pH 8.8) and 1 m*M* ADP-glucose were held at 37°C for 4 h.

Detection of phosphorylase

Phosphorylase activity became visible, when gel stripes were incubated in 50 m*M* tris–citrate buffer, (pH 6.2), containing 2.5 m*M* 1,4-dithio-*DL*-threitol, 10 m*M* glucose 1-phosphate and 5 m*M* EDTA in a test tube at 37°C for 1 h.

RESULTS AND DISCUSSION

The present work describes a simultaneous and sensitive technique for staining and detection of Q-enzyme, R-enzyme, soluble starch synthase, phosphorylase and α-amylase on starch–PAGE. In comparison with earlier works, the present one is more effective concerning Q-enzyme activity due to shorter reaction times and sharp stained bands on the gel. Our intention was to develop a simple and reliable method for activity staining which permits the detection of various enzymes of the starch me-

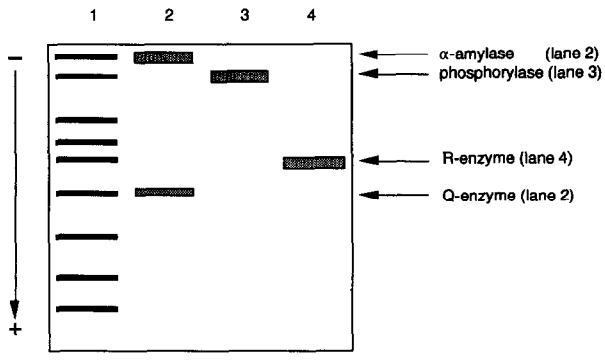

Fig. 1. Simultaneous staining of Q-enzyme, α-amylase, phosphorylase and R-enzyme activity on starch–PAGE. Aliquots (50 μl each) of crude potato juice were applied to the gel. After electrophoretic separation, the gels were incubated in the substrate solutions as described in the text. Lanes: 1 = Protein stain of the corresponding crude potato juice; 2 = simultaneous iodine stain of Q-enzyme and α-amylase; 3 = phosphorylase activity; 4 = iodine stain of R-enzyme activity.

tabolism on the same gel plate. In this manner it is possible to stain crude supernatants as well as purified enzyme extracts. In Fig. 1 the activity stains of Q-enzyme, α-amylase, phosphorylase and R-enzyme are shown. Lane 1 presents the Coomassie stain of the whole potato extract. Lane 2 shows the simultaneous iodine staining of Q-enzyme and α-amylase from a crude potato extract subjected to electrophoresis. Q-enzyme hydrolyses α-(1→4)-linkages with following chain transfer including the formation of α-(1→6)-linkages on suitable acceptor chains. The transferred short-chains give a sharp red band on the blue background basing on the shift of λ_{max} of the iodine–starch complex to shorter wavelengths and can be clearly distinguished from α-amylase activity which appears as clear and transparent bands on the gel. To avoid confusion between Q-enzyme and α-amylase activity from potatoes in starch gels of different acrylamide concentration, BSA can be used as a reference protein in the Coomassie stain (not shown). As we reported previously [29], Q-enzyme and BSA slightly differ in the relative mobilities in SDS-PAGE as well as in native PAGE using 15% acrylamide. Samples from either plant or animal tissue can be tested for enzyme activity in a short time. Q-enzyme activity could be detected even with the naked eye as a thin white band before iodine staining. This observation leads to the opinion that the transferred short-chains cause a band of higher starch density in the gel. To support evidence for this result the same stripe was stained again with iodine solution after three weeks rinsed in water to demonstrate that the red stained short-chains are all attached by newly synthesized α-(1→6) bonds to the starch in the gel. Secondly, by this observation we could confirm that branching enzyme is an enzymic entity. Decrease in reducing power in samples containing starch and the cut out Q-enzyme band could be observed by the modified Somogyi–Nelson method [31,32].

On addition of glucose 1-phosphate to the substrate phosphorylase activity (lane 3) becomes visible. A white band of elongated α-1,4-glucan chains synthesized by phosphorylase activity could be detected, when gel stripes are incubated in 50 m*M* tris–citrate buffer, (pH 6.6) containing 1 m*M* dithiothreitol, 20 m*M* glucose 1-phosphate and 5 m*M* EDTA at 37°C for 2 h. Using the same buffer system (pH 7.0) as described for Q-enzyme staining, a simultaneous staining of phosphorylase is possible although increasing reaction time is observed. The reaction time of the activity staining of synthesized α-(1→4)-bonds within one hour strongly varies with the amount of glucose 1-phosphate added in the substrate solution.

Lane 4 shows the activity stain of R-enzyme.The enzyme activity hydrolizes the α-(1→6) bonds and leads to light blue bands on the blue backgound of the iodine stained gel. The split off chains appear to be of a rather short length able to diffuse out of the gel.

The intention was to harmonize the pH value of the incubation medium corresponding to the different pH-optima of the enzymes in the stain. For this reason starch synthase activity which demands higher pH values could not be stained simultaneously with the other enzymes. Current studies show that Q-enzyme is inhibited at pH values from 8.5 to 9.0 [23]. Fig. 2, lane 1 shows the protein stain of crude potato extract subjected to starch-PAGE. Lane 2 presents the phosphorylase activity at pH 6.6 and in lane 3 the soluble starch synthase activity can be seen, presenting two white bands of synthesized α-(1→4) chains on the gel. One band corresponds to the phosphorylase activity (in lane 1). This observation has been confirmed by paired-ion reversed-phase high-performance liquid chromatography when gel stripes are cut out horizontal

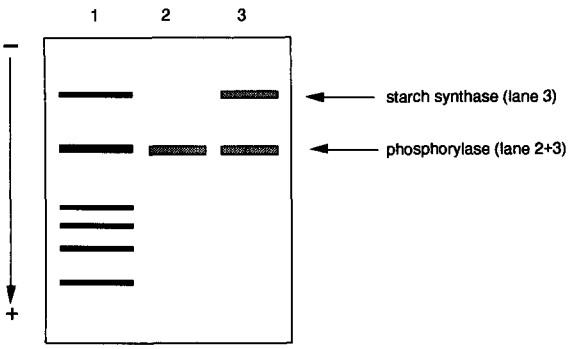

Fig. 2. 1 = Coomassie stain of a crude potato extract. 2 = Band of synthesized α-(1→4) chains in the gel stripe show the phosphorylase activity. Gel stripes were incubated in 50 m*M* tris–citrate buffer (pH 6.2), containing 2.5 m*M* 1,4-dithio-DL-threitol, 10 m*M* glucose 1-phosphate and 5 m*M* EDTA in a test tube at 37°C for 1 h. 3 = Primed soluble starch synthase activity and phosphorylase activity. The reaction was performed in a test tube containing 100 m*M* NaOH–glycine buffer (pH 8.8), and 1 m*M* ADP-glucose at 37°C (see Experimental).

and incubated with ADP-glucose in the presence of suitable primers (unpublished data).The results lead to the conclusion that phosphorylase can use ADP-glucose or decomposition products as a substrate. The above results imply that ADP-glucose could be metabolized to glucose 1-phosphate prior to incorporation into starch by phosphorylase. The question arises if the presence of ADP-glucose pyrophosphorylase (glucose 1-phosphate adenyltransferase, EC 2.7.7.27) in the extract could participate in this reaction. This enzyme is believed to play a key regulatory role in the gluconeogenesis which equally operates in amyloplasts and chloroplasts [33].Worth to be mentioned is the fact that this type of activity becomes visible on the gel with ADP-glucose as a glycosyl donor whether inorganic phosphate in the incubation medium is present or not.

The above results lead to the conclusion that in comparison with several other staining techniques the present system might be expected to give a more comprehensive and extensive activity staining of the discussed enzymes. The methods are appropriate to prove the activity staining of some important enzymes of the glucan metabolism. In this manner highly purified enzymes can be isolated in semipreparative scale from gels with a thickness of 3 mm as we use in our laboratory for enzymic synthesis of *de novo* products. Thus, the method may be helpful for the detection, purification and identification of enzyme activities in plant or animal tissues in the study of starch biosynthesis without the need of other time-consuming purification steps.

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