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Short Report

A Novel Method to Detect β-Cyclodextrin Glucosyl Transferase (β-CGTase) Activity on Polyacrylamide Gels

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β-cyclodextrin glucosyl transferase (β-CGTase) hydrolyses starch to produce β-cyclodextrin by transglycosylation (cyclization) activity. The conventional method for detection of β-CGTase activity is based on detecting starch hydrolysis by iodine staining. This method reveals all amylolytic enzymes, but can not discriminate them. In the present study, we introduce a new method for specific detection of β-CGTase activity and its specific product i.e. β-cyclodextrin by polyacrylamide gel. In this method, solution containing β-CGTase is subjected to electrophoresis on 10% polyacrylamide gel. Then, the gel is covered with an indicator gel containing phenolphthalein, soluble starch, and agar. After a short incubation, sodium carbonate solution is added and the positive result is indicated by the emergence of a colorless band in the red context of the indicator gel. Since the production of β-cyclodextrin via cyclization is unique to β-CGTase, the emergence of clear bands is indicative of β-CGTase presence. However, in conventional starch-iodine method, all amylolytic enzymes including β-CGTase give positive results. Therefore, for detection of β-CGTase, the phenolphthalein indicator gel method, compared to the starch-iodine method, is more specific. *Iran. Biomed. J. 9 (2): 87-90, 2005*

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INTRODUCTION

Beta cyclodextrin glucosyl transferase (β-CGTase) (E.C. 2.4.1.19) is an enzyme produced by some strains of bacteria including members of the genera Bacillus, Paenibacillus, Klebsieella, Anaero-bacterium, etc. [1-4]. This enzyme catalyzes the reaction of transglycosylation. Cyclization is a special form of transglycosylation that leads to the production of cyclodextrins from starch [4]. Cyclodextrins are cyclic polymers of glucose and have wide applications in pharmaceutical, cosmetics, and food industries [5-7]. Basic and applied research work on this enzyme sometimes necessitates the detection of CGTase activity of protein bands seperated by electrophoresis on polyacrylamide gel [8]. It is also important to differentiate this enzyme from other concomitant amylolytic enzymes separated by electrophoresis. For example, at each step of enzyme purification process, the efficacy of the process can be monitored and evaluated qualitatively by electrophoresis. Iodine-staining is commonly used for detection of β-CGTase activity. In this method, the gel is immersed in a solution containing starch, and after about 30 minutes iodine is applied onto the polyacrylamide gel. The appearance of clear bands in the context of blue color is indicative of starch hydrolysis. This method is non-specific because various starch-hydrolyzing enzymes namely α- and β- amylases, glucoamylase, pullulanase and, β-CGTase, give positive results [9]. In order to differentiate β-CGTase from other amylolytic enzymes, the clear bands are cut from the gel, followed by extraction of the enzyme out of the gel and performing the specific reaction to identify the enzyme in the test tube [8].

In the present study, we introduced a specific method to identify and differentiate β-CGTase activity from other amylolytic enzymes on

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polyacrylamide gel in one step. This new method is based on the detection of the specific product of β-CGTase, β-cyclodextrin, that forms inclusion complex with phenolphthalein and results in decoloration of the dye.

MATERIALS AND METHODS

Chemicals. Alpha-amylase and glucoamylase enzyme samples were obtained from Novo Nordisk (Denmark). Soluble starch was purchased from Merck (Germany). All other chemicals were purchased from Sigma (USA).

CGTase preparation. A new alkalophilic *Bacillus* sp. (strain IROST-79) isolated from rotten potato samples was cultivated in 50 ml of Horikoshi II liquid medium containing 1% soluble starch, 0.5% yeast extract, 0.5% peptone, 0.02% $MgSO_4.7H_2O$, 0.1% K_2HPO_4 and 1% Na_2CO_3 with a final pH of 10 [1]. The cultures were incubated at 37℃ in a shaker incubator (180 rpm) for 24 h. Then, the supernatant containing β-CGTase was collected after centrifugation at 10,000 ×g for 20 min. The proteins of this supernatant were precipitated with saturated ammonium sulfate. Dialysis against phosphate buffer (pH 8) was done to solubilize the proteins. Then, the dialyzed solution was double concentrated by polyethylene glycol.

Electrophoresis. Non-denaturing discontinuous PAGE was performed with 10% polyacrylamide gels according to Davis [10] but in slab gel. In two identical sets of three consecutive wells, samples of β-CGTase preparation, α-amylase, and gluco-amylase were applied, respectively. The gel was washed with 0.2 M phosphate buffer (pH 8) and was cut between the two sets and each one was subjected to a different staining method.

Iodine staining method. One half of the gel was incubated in 3% soluble starch at 37℃ for 30 minutes. Then, the gel was washed with distilled water, and stained with a solution containing 0.1% I_2 in 1% KI. Development of a clear band in the blue context of the gel is indicative of amylolytic activity.

Phenolphthalein indicator gel method. Just before the completion of the electrophoresis, the indicator gel was prepared by mixing 0.24 g soluble starch, 0.14 g agar in 16 ml of 0.2 M phosphate buffer (pH 8). After the mixture, 0.5 ml of 0.4% phenolphthalein was added and the whole mixture was cooled about 50°C. The indicator gel was poured uniformly on the second half of the polyacrylamide gel and was allowed to solidify. After a 5-minute incubation at 37℃, the indicator gel was flooded with a 0.1% sodium carbonate solution until the context of the gel turned into red except for a colorless band indicative of β-CGTase activity.

Coomassie blue staining method. After visualization of β-CGTase activity, the indicator gel was gently removed from the top of the polyacrylamide gel and discarded. Polyacrylamide gel was subjected to conventional Coomassie blue staining method.

RESULTS

The conventional method for detection of CGTase activity is based on detecting starch hydrolysis by starch-iodine method. This method is non-specific because all of starch-degrading enzymes, regardless of their specific reactions, show similar results and thus are indistinguishable. However, the phenol-phthalein indicator gel method is based on the detection of the specific product of β-CGTase, that is, β-cyclodextrin. β-cyclodextrin forms inclusion complex with phenolphthalein and results in decoloration of the dye. Figure 1 shows the results of starch-iodine staining of polyacrylamide gel. All three enzymes: α-amylase, glucoamylase, and β-CGTase gave positive results with conventional method. Interestingly, two bands with starch hydrolyzing activity were visible in the β-CGTase lane. However, only β-CGTase produced a positive result when stained with phenolphthalein indicator gel method (Fig. 2). In order to rule out the possibility that the negative results of α-amylase and glucoamylase in phenolphthalein indicator gel method have been due to low amount of the loaded samples, Coomassie blue staining was used. Figure 3 shows the presence of high amounts of these two enzymes. Although the amount of loaded samples in α-amylase and glucoamylase lanes was more than the β-CGTase lane (Fig. 3), these two enzymes didn't produce the colorless positive band with phenolphthalein indicator gel (Fig. 2).

DISCUSSION

β-CGTase catalyzes the production of β-cyclodextrin from starch. cyclodextrins are homologous series of non-reducing and cyclic D-glucose polymers varying in size from 6-12 glycosyl ring structures [11]. The center of cyclodextrins has a non-polar hydrophobic cavity, that easily form inclusion complexes with organic and inorganic compounds. This property of cyclodextrins has been utilized in pharmaceutical, cosmetics, and food industries. The phenomenon of inclusion complex formation between β-cyclo-dextrin and phenolphthalein resulting in a colorless complex has been used formerly. Vikmon used this phenomenon to develop a spectro-photometric method to measure β-cyclodextrin in solutions [12, 13]. On this principle, Park *et al.* [14] designed a screening medium to isolate β-CGTase producing bacteria from soil samples. In the present study, we used an indicator gel containing phenolphthalein to detect β-CGTase activity on polyacrylamide gels specifically and to discriminate this protein band from other starch-hydrolyzing enzymes in one step.

The general and non-specific iodine-staining method is indicative of starch hydrolysis, therefore, it can not discriminate between any two amylolytic

Fig. 1. Iodine staining. Clear bands are indicative of starch hydrolysis. Lane 1, cyclodextrin glucosyltransferase; lane 2, α-amylase and lane 3, glucoamylase.

Fig. 2. Phenolphthalein staining. A sharp clear band is indicative of cyclodextrin production. There are not any bands in α-amylase and glucoamylase lanes. Lane 1, cyclodextrin glucosyltransferase; lane 2, α-amylase and lane 3, glucoamylase.

Fig. 3. Coomassie blue staining. Indicator gel is taken off from the electrophoretic gel and the latter is stained with Coomassie blue. Lane 1, cyclodextrin glucosyltransferase; lane 2, α-amylase and lane 3, glucoamylase.

enzymes [9]. Moreover, it is known that most of the β-CGTase-secreting bacterial strains produce other amylolytic enzymes, such as α-amylase [15]. In these cases, using iodine staining alone reveals more than one band that may be misinterpreted as β-CGTase isozymes. Some investigators [8], cut the gel and extract the enzyme to verify β-CGTase activity.

Our results showed two bands in CGTase lane when electrophoretic gel stained with starch-iodine method. However, staining with specific phenolphthalein indicator gel method revealed only one band indicative of β-CGTase presence. Therefore, this new alkalophilic bacillus produces both β-CGTase and an amilolytic enzyme and the phenolphthalein indicator gel method discriminates between them. The other advantage of our method is using starch agar gel in place of starch solution. The agar overlay method has the advantage of bringing the reagents into contact with the enzyme and also prevents diffusion of soluble β-cyclo-dextrins. Thus, β-cyclodextrin becomes concentrated *in situ* leading to increased sensitivity and a sharper band and allows precise cutting of the enzyme band when applied to preparative gels.

The phenolphthalein indicator gel method also allows the reintroduction of polyacrylamide gel into Coomassie staining after visualization of β-CGTase activity. Therefore, this method is very simpler to identify β-CGTase in comparison with the general iodine stain method.

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