A Rapid Screening Method for Alkaline β-Cyclodextrin Glucanotransferase using Phenolphthalein—Methyl Orange-containing-sol中 Medium

Cheon Seok Park, Kwan Hwa Park and Seung Ho Kim*

Department of Food Science & Technology, Seoul National University, Suwon 440-744, Korea

*Korea Food Research Institute, c/o KAIST, P.O. Box 131, Cheongryang, Seoul, Korea

Received October 25, 1988

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) acts on starch, producing α-, β- and γ-cyclodextrins (CDs). The molecular structure of CDs has a hollow cavity of a specific volume, in which CDs are able to form inclusion complexes with other substances.

CGTase-producing microorganisms have been isolated from soil on agar medium containing corn starch, on which clear zones were formed.1-2) In this screening test, however, CGTase activity was indistinguishable from amylase activity, so additional methods, specific for CGTase activity, such as high performance liquid chromatography (HPLC),3) the glucoamylase method,4) spectrophotometric method5) or trichloroethylene method,6) were necessary after the incubation of isolates. In this work we developed a simple and rapid screening method by which CGTase can be specifically detected on agar medium.

Taguchi7) found that phenolphthalein was transformed into a colorless dianion within the cavity of β-CD. Recently, CGTase activity was determined on the basis of the reduction of the color intensity of phenolphthalein under alkaline conditions.5) It has also been reported that various dye molecules, such as congo red and methyl

<table>
<thead>
<tr>
<th>Enzyme and microorganism</th>
<th>Assay methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iodine test*</td>
</tr>
<tr>
<td></td>
<td>Phenolphthalein—β methyl orange medium</td>
</tr>
<tr>
<td></td>
<td>Trichloroethylene test</td>
</tr>
<tr>
<td></td>
<td>High-performance—liquid chromatography (mg β-CD/5 ml culture broth)</td>
</tr>
<tr>
<td>Culture broth of B. macerans</td>
<td>++</td>
</tr>
<tr>
<td>Culture broth of B. circulans ATCC 21783</td>
<td>+ +</td>
</tr>
<tr>
<td>Culture broth of CS-2, isolated from soil</td>
<td>+ +</td>
</tr>
<tr>
<td>Termamyl (Novo)</td>
<td>+ + +</td>
</tr>
<tr>
<td>Taka-amylase (Miles Lab.)</td>
<td>+ + +</td>
</tr>
<tr>
<td>Glucoamylase (Miles Lab.)</td>
<td>-</td>
</tr>
</tbody>
</table>

* + + +, large; + +, medium; +, small; -, not detected.

* 10 μl of each enzyme solution was spotted on 2% soluble starch medium II, followed by incubation, and then an iodine solution (0.203 g I₂ + 5.202 g KI/100 ml distilled water) was poured to determine the sizes of the hollow zones.

* 10 μl of each enzyme solution was spotted on phenolphthalein—methyl orange medium II, followed by incubation to determine the sizes of the hollow zones.

* The enzyme solution was diluted successively twice with 0.2 M Borax buffer (pH 8.5) to obtain dilution stages of 2¹, 2², 2³, ··· 2ⁿ. 1 ml of each diluted enzyme solution was mixed with 5 ml of a 2% soluble starch solution, followed by incubation at 40°C for 48 hr. 2.5 ml of trichloroethylene was then added to the reaction mixture with vigorous shaking. The final dilution at which there was appreciable precipitation was taken as the activity.

* 1 ml of each enzyme solution was reacted with 5 ml of a 2% soluble starch solution and then 10 μl of the mixture was injected onto the HPLC column.
Fig. 1. Assaying of Cyclodextrin Glucanotransferase (CGTase) Activity on Phenolphthalein-Methyl Orange-containing Solid Medium.

Bacterial cells were patched on the plate, followed by incubation for 24 hr at 37°C. The presence of a hollow zone indicates CGTase activity. A, B. macerans; B, B. circulans (ATCC 21783); C, CS-2 isolated from soil.

orange, can be included in the cavity of CD molecules. We investigated as to whether or not these properties of CD molecules could be applied to the detection of CGTase activity in agar medium containing a mixture of dyes.

For the screening test, Horikoshi's medium II was modified to contain 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K$_2$HPO$_4$, 0.02% MgSO$_4$.$\times$H$_2$O, 1% Na$_2$CO$_3$, 1.5% agar, 0.03% phenolphthalein and 0.01% methyl orange (all concentrations in w/v in distilled water). To ensure that CDs can form inclusion complexes with phenolphthalein in the solid medium, 10 μl each of 0.01% (w/v, in 0.2 m NaOH–glycine buffer, pH 9.0) α-, β- and γ-CD solutions was spotted on the modified medium. α-CD did not give a noticeable transparent zone, but the spots of the β- and γ-CDs were surrounded by appreciable yellowish hollow zones. A reduction in the color intensity was not observed for α-CD, since the complex of α-CD and phenolphthalein was not easily formed.

Culture broth of the bacterial stock strains, Bacillus macerans, Bacillus circulans (ATCC 21783) and CS-2 (an unidentified bacterium, isolated from soil in our lab.) (all three incubated in Horikoshi's medium II, without agar, at 37°C for 48 hr), all of which are known to exhibit CGTase activity, was applied to an appropriate medium (as described in Table I) and then incubated at 37°C for 6 hr. Termamyl (NOVO), Taka-amylase (Miles Lab.) (both are kinds of α-amylase) and glucoamylase (all 1.0% (w/v), in 0.2 m NaOH–glycine buffer, pH 9.0) were also spotted and incubated in the same way. As shown in Table I, all the spots, except those of glucoamylase, showed clear zones in the iodine-starch test, whereas only broth containing CGTase activity gave yellow hollow zones. The possibility that acid-producing microorganisms changed the color of phenolphthalein in colorlessness by producing acids, was examined by adding methyl orange to the medium. The color of methyl orange will change from orange to red with acid-forming bacteria, so that one can distinguish CGTase activity from the actions of acids produced by some microorganisms.

The bacterial stock strains were examined on the agar medium containing phenolphthalein–methyl orange. For colonies grown on plates, 24 hr incubation at 37°C was sufficient for the appearance of clear zones of 1 to 2 cm in diameter around the colonies. All of the stock strains examined exhibited CGTase activity, giving clear zones (Fig. 1). When the diameters of the hollow zones around culture broth and colonies were compared with the results of the trichloroethylene precipitation test and HPLC analysis, strong correlation was observed (Table I and Fig. 1), which indicates that the size of a transparent zone is a good measure of the CGTase activity.
The suitable combination and concentrations of phenolphthalein and methyl orange, in the range of 0.01 ~ 0.05\% (w/v, in medium II), were examined on the basis of the clearness of the transparent zone and the growth of the microorganisms, and 0.03\% phenolphthalein and 0.01\% methyl orange were found to be best. Since CGTase activity can be determined specifically and quantitatively in the isolation stage, the screening test should save a lot in terms of energy, time and material, compared to hitherto used conventional screening methods for alkaline $\beta$- and $\gamma$-CGTase-producing microorganisms. The use of phenolphthalein and methyl orange as indicators for CGTase activity in an agar medium provides the basis for a rapid and sensitive screening test for $\beta$- and $\gamma$-CD-producing bacteria. Even though this paper dealt only with alkaline conditions, the method can be easily extended to neutral and acidic conditions by changing the dye(s) and/or isolating medium.

References