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Note

An environmentally acceptable method for assaying the inhibition of α-amylase induction

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A facile α-amylase induction inhibition assay method was investigated. Induced α-amylase was determined using a microplate filled with a gel containing rice starch and Gellan gum. Embryoless barley seeds were incubated with GA_3 (10^{-7} M) and appropriate ABA. Diluted incubation fluid was dispensed to the wells and the change of absorbance was measured. Induced α-amylase was calculated from the change of the absorbance. The results were almost equal to those of the Somogyi–Nelson method. As noxious wastewater containing heavy metal ions does not occur in this experiment, this method can be an environmentally acceptable assay method for ABA analogs.

Keywords: α-amylase quantification, gibberellic acid, abscisic acid, α-amylase induction inhibition assay.

Introduction

When barley seeds germinate, gibberellic acid (GA_3), first synthesized in the embryo, is transported to the aleurone layer and induces α-amylase biosynthesis in the aleurone layer. This process is specifically inhibited by plant hormone abscisic acid (ABA).[1] This inhibition of α-amylase biosynthesis occurs at the transcription stage and the inhibition of α-amylase mRNA synthesis is recognized as a specific activity of ABA.[2] This inhibition of α-amylase induction is used as a specific bioassay method for ABA and its analogs.[3] The Somogyi–Nelson method has been widely used to evaluate the amount of induced α-amylase by measuring the quantity of reducing sugars in the culture fluid.[4,5] Although the method has excellent sensitivity for reducing sugars, wastewater including arsenic and copper compounds arises in the experimental procedure.[6]

In 1998, Satoyama *et al*. reported a convenient method for measuring α-amylase activity. The principle of this method is based on the turbidity change caused by the hydrolysis of precipitated starch by added α-amylase.[7,8] This method seems valid for our purpose since it enables us to deal with many samples automatically and to exclude the use of toxic metal salts. We tried to apply this method for the α-amylase induction assay. In this paper, we will discuss the improved α-amylase measuring method and its application in the research of ABA analogs.

Materials and Methods

1. *Materials and reagents*

Gibberellic acid (GA_3) and (*S*)-abscisic acid were obtained from Kyowa Hakko Co. Ltd. and the barley seeds (*Hordeum vulgare* L. cv. Ichibanboshi) were from Kumamoto Prefectural Agricultural Research Center. All other reagents and solvents were from Wako Pure Chem. Industries (Osaka).

2. *Preparation of the substrate plate*

2.1. *Preparation of the substrate plate solidified with agar*

The substrate plate solidified with agar was prepared as follows: rice starch (1.5 g) and agar (1.5 g) were dissolved in 100 ml of citrate buffer (0.1 M, pH 6.0) in a boiling water bath until the suspension became a clear solution. The hot solution was dispensed into the wells of the microplate, which was left at room temperature until the solution solidified. The absorbance of each well at 620 nm was measured using a microplate reader, NJ-2100. Each experiment was carried out for 32 runs, and then the microplate was sealed with tape and stored at 4°C in a refrigerator.

2.2. *Preparation of the substrate plate solidified with Gellan gum*

Rice starch (1.5 g) was dissolved in 50 ml of citrate buffer (0.1 M, pH 6.0) in a boiling water bath. To this solution, an equal volume of 1.2% Gellan gum hot solution was added with stirring at 90°C. Calcium lactate solution (1 ml: 200 mM) was added to this hot solution and then 100 μl of the solution was dispensed into the wells of the microplate. The microplate was left at room temperature until the mixed solution solidified, and the absorbance of each well at 620 nm was measured using a microplate reader (ImunoReader NJ-2100: InterMed Co., Ltd.). Each experiment was carried out for 32 runs, and then the microplate was sealed with tape and stored at 4°C in a refrigerator.

3. *Preparation of the calibration curve for α-amylase*

α-Amylase solutions (50 μl containing 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1.0 units, respectively) were added to the wells of the substrate plate with cooling on crushed ice. The sample plate was warmed in a water bath (37°C) for 3 min and absorbance at 620 nm was measured using a microplate reader. The sample plate was incubated at 37°C for 90 min and the absorbance of each

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well was measured every 30 min. The measurement was carried out for 32 runs. The difference of absorbance (ΔAbs) was defined as follows.

ΔAbs = Absorbance after 30 min − Absorbance after 90 min.

4. Determination of optimum GA_3 concentration for the α-amylase induction inhibition test using Hordeum vulgare L. cv. Ichibanboshi

Barley seeds were suspended in 30 ml of 50% sulfuric acid and the suspension was left in the refrigerator for 3 hr at 4°C. After sterilization, 50% sulfuric acid was discarded and the seeds were washed with ice-cooled sterilized water (30 ml) 3 times by shaking for 15 min. This washing was repeated 19 times and then the seeds were soaked in 30 ml of sterilized water and left in a refrigerator at 4°C for 15 hr.

Petri dishes equipped with two sheets of filter paper were sterilized at 180°C for 2 hr. The ABA solutions (5 ml: 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M) were added to the Petri dishes, respectively, and left on a clean bench to dry the solution. Petri dishes for the blank test and positive control test were used for the next step without the addition of ABA solution. GA_3 solution (5 ml: 10^{-6} M, 10^{-7} M and 10^{-8} M) in 50 ppm streptomycin sulfate solution was then added to the Petri dishes, respectively. The dishes for the blank test received 5 ml of 50 ppm streptomycin sulfate solution.

Barley seeds soaked at 4°C for 15 hr were halved transversely. The embryo-less cut sections were placed downward on to the filter paper and incubated for 48 hr at 30°C in the dark. After incubation, the incubation fluid was diluted with distilled water at 1/20. The diluted incubation fluid (50 μl) was dispensed to the wells of the previously prepared substrate plate cooling on the crushed ice. The sample plate was then warmed in the water bath (37°C) for 3 min and absorbance at 620 nm was measured by using the microplate reader. The sample plate was incubated at 37°C for 90 min and the absorbance of the each well was measured using the microplate reader every 30 min. The induced α-amylase unit was calculated by the value of ΔAbs using the calibration curve. Measurement was carried out for 24 runs.

Results and Discussion

1. Comparison of gelatinizer

We traced the α-amylase quantifying protocol described by Satoyama *et al*., using purchased α-amylase, rice starch and agar. The dispensation of the substrate solution was not easy since the substrate agar solution solidified in the dispensing process and it was difficult to level the injection volume in each well. Table 1 shows that the absorbance at 620 nm of the agar-rice starch gel was 0.727 and the standard deviation was 0.031. In order to improve the difficult dispensation process and low transmittance, the gelatinizer was changed to Gellan gum. Gellan gum is a transparent gelatinizer and is usually used as the culture support of plant tissue culture. This change was very effective not only for easy dispensation of the substrate solution but also to improve the transmittance of the substrate gel, as shown in Table 1. The average value of the absorbance and the standard deviation of the absorbance were 0.474 and 0.013, respectively.

Table 1. Average optical density and standard deviation of microplate wells[a)]

	AOD	SD
Blank cell	0.037	0.004
1.5 % Rice Starch Solidified with Agar (1.5 %)	0.727	0.031
1.5 % Rice Starch Solidified with Gellum gum (0.6 %)	0.474	0.013

[a)] AOD: average optical density, SD: standard deviation. Each experiment was carried out for 32 runs.

2. Preparation of the calibration curve for α-amylase quantification

In this experiment, the absorbance change of the wells sometimes differed in the first 30 min. This phenomenon may be caused by the non-uniform diffusion of the added α-amylase in the gel; therefore, the difference in the absorbance after 30 min and that after 90 min was used.

The correlation between the added α-amylase quantity and the difference in absorbance (ΔAbs) are shown in Fig. 1. The difference of the absorbance was in proportion to the α-amylase quantity in the range of 0.001 units and 1.0 unit, and the correlation coefficient calculated by the least squares method was 0.9889. This graph was used as the calibration curve for calculating the induced α-amylase quantity in the α-amylase induction inhibition assay.

3. α-Amylase induction inhibition assay

The barley cultivar Ichibanboshi was used in this experiment. To find the optimum GA_3 and abscisic acid concentrations for this

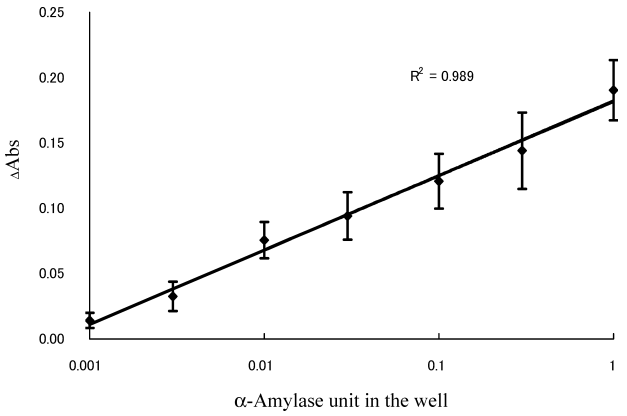

Fig. 1. Correlation between the applied α-amylase unit and the decrease in absorbance. The decrease of absorbance was calculated by subtracting the absorbance after 90 min reaction from the absorbance after 30 min reaction, respectively.

Table 2. Induced α-amylase units in the culture medium

ABA conc. (M)	GA_3 conc. (M)	ΔAbs[a)]	Induced α-amylase[b)] (unit/well)	α-Amylase induction ratio (%)
0 (Positive control)	10^{-6}	0.108	1.12	100.0
0 (Negative control)	0	0.015	0.06	0.0
1×10^{-6}		0.088	0.50	39.3
3×10^{-7}		0.104	0.95	84.0
1×10^{-7}	10^{-6}	0.105	0.99	87.7
3×10^{-8}		0.106	1.03	91.5
1×10^{-8}		0.107	1.07	95.3
0 (Positive control)	10^{-7}	0.101	0.84	100.0
0 (Negative control)	0	0.006	0.02	0.0
10^{-5}		0.009	0.02	0.2
10^{-6}		0.051	0.11	11.3
10^{-7}	10^{-7}	0.070	0.24	26.9
10^{-8}		0.085	0.44	51.2
0 (Positive control)	10^{-8}	0.057	0.14	100.0
0 (Negative control)	0	0.009	0.02	0.0
10^{-5}		0.011	0.02	1.4
10^{-6}		0.015	0.03	5.0
10^{-7}	10^{-8}	0.015	0.03	5.0
10^{-8}		0.032	0.05	25.6

[a)] ΔAbs = Absorbance after 30 min − Absorbance after 90 min. [b)] The amount of induced α-amylase was calculated from the difference of absorbance (ΔAbs) per hour using the calibration curve. ΔAbs values represent the means for 24 runs.

cultivar, embryo-less cut sections were treated with 10^{-6} M, 10^{-7} M and 10^{-8} M of GA_3 to induce α-amylase synthesis. The sections were simultaneously treated with 10^{-5} M–10^{-8} M abscisic acid to inhibit α-amylase induction. The α-amylase induction inhibition assay results are shown in Table 2. It is apparent that 10^{-6} M GA_3 treatment was too severe since ABA could not inhibit α-amylase induction even at the concentration of 3×10^{-7} M. On the other hand, α-amylase induction by 10^{-8} M GA_3 was too weak since only a small amount of α-amylase was induced in the positive control test. Induction by 10^{-7} M GA_3 is therefore the most suitable. Table 2 indicates that the induced α-amylase ratios when embryo-less barley seeds were treated with 10^{-7} M GA_3 and 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M ABA were 0.2, 11.3, 26.9, and 51.2%, respectively. It is apparent that a concentration-dependent inhibition of GA_3-induced α-amylase induction by ABA is clearly observed by this method.

The Somogyi–Nelson method has been widely used to determine inhibiting activity. This method quantifies the amount of reducing sugars in the culture fluid, whereas the new method determines the induced α-amylase activity; therefore, it seems that the results obtained by the two methods do not completely agree. We previously reported that the induction of α-amylase by 5×10^{-8} M GA_3 was inhibited by 1.1×10^{-5} M and 3.3×10^{-5} M of ABA application by 98% and 100%, respectively, using the Somogyi–Nelson method.[5)] These results were almost the same as in this experiment. Consequently, it can be deemed that the new method has almost equal detectability as that of the Somogyi–Nelson method.

Moreover, the regulation of wastewater including toxic substances has intensified recently. We need to devise new methods without accompanying toxic wastewater even in research and development. As noxious wastewater containing heavy metal ions does not occur in this method, which is suitable for large-scale processing by automation, it seems to be an environmentally acceptable assay method for the research of ABA analogs and α-amylase itself.

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