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Quantitative β-glucuronidase assay in transgenic plants

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Abstract

Several factors influencing reliability of the quantitative fluorimetric β-glucuronidase (GUS) assay in transgenic plant tissue have been investigated. We obtained linear dependence of fluorescence on both the duration of hydrolysis and the extract concentration. The stability of the enzyme in the homogenate was fairly high, the same as the stability of the substrate solution and of the final reaction product. The modification of the extraction/incubation buffer was proposed, resulting in several times higher activity in comparison with original procedure.

Introduction

The transformation of plant cells by *Agrobacterium tumefaciens* carrying the β-glucuronidase (GUS) gene from *Escherichia coli* and the proof of its expression frequently have been studied. Relatively little attention, however, has been payed to quantitative assay of GUS activity in transformed cells. The aim of the present communication was to fill this gap.

Material and methods

The experiments were done using leaf tissue of tobacco (*Nicotiana tabacum* L. cv. Samsun) transformed two years ago by *Agrobacterium tumefaciens* strain LBA 4404 with pBI 121.1 plasmid carrying chimeric GUS and NPT II (neomycin phosphotransferase II, *i.e.* kanamycin resistance) genes (Jefferson 1988). After regeneration on Murashige and Skoog (MS) medium with growth regulators and antibiotics and the initial cultivation on plain MS media only with antibiotics, the plants were kept on MS media and subcultured every month (see Ondřej *et al.* 1991). The leaves of *corresponding* ontogenetic stage were assayed.

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We modified the fluorescence method with 4-methyl umbelliferyl β-D-glucuronide of Jefferson (1987). 25 mg of leaf tissue was homogenized in 1 cm^3 of extraction/incubation (*e/i*) buffer (containing 245 cm^3 of H_2O, 85 cm^3 of 10^{-1} M NaH_2PO_4, 165 cm^3 of 10^{-1} M Na_2HPO_4, 185 mg Na_2 EDTA, 345 mm^3 β-mercapto-ethanol, 5 cm^3 10 % Triton X-100) in glass homogenizer of *Potter-Elvehjem* type. 30 mm^3 of the homogenate was added to 450 mm^3 of incubation medium [2 mg 4-methylumbelliferyl β-D-glucuronide (*Sigma*) dissolved in 50 cm^3 of *e/i* buffer]. After 20 min at room temperature 400 mm^3 of this reaction mixture was transferred into 3.5 cm^3 of stop-solution (0.2 M Na_2CO_3). In order to evaluate spontaneous decomposition of the substrate (blank) 30 mm^3 of *e/i* buffer was used instead of the homogenate. The fluorescence was assayed using *Turner 111* fluorometer, 3.5 cm^3 cuvette, excitation by radiation of wavelength 365 nm, emission measured at 455 nm (gray absorption filter - 1 % range, *Kodak-Wratten 96* was used to attenuate the signal). The results are expressed as working units directly measured or recounted. In the present experimental system the fluorescence of 10^{-7} M 4-metylumbelliferone (*Clontech*) dissolved in the fluid obtained by mixing *e/i* buffer and stop solution as given above was 20.33 (s_x=1.85).

Results and discussion

Using the above mentioned procedure we obtained linear dependence of fluorescence on both the duration of the hydrolysis (Fig. 1) and the sample quantity (extract concentration, Fig. 2).

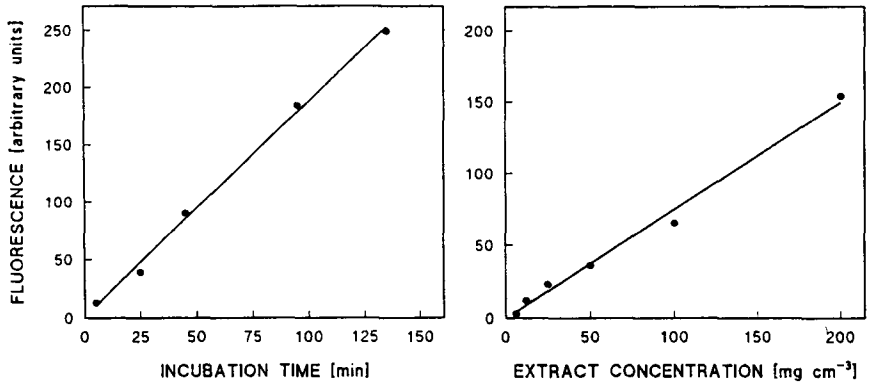

Fig.1. The dependence of activity on the incubation time.
Fig.2. The dependence of activity on the concentration of the extract.

Further we tested the dependence of activity on the composition of incubation medium (Fig. 3). The highest activity was found in the medium containing Triton X-100, Na_2EDTA and β-mercaptoethanol. That is why we chose this medium to be used in our standard procedure, omitting sodium lauroyl sarcosine (sarkosyl; 0.1 %

v/v). Considerable spontaneous decomposition of substrate in plain buffer must be emphasized in comparison with *e/i* buffer (about ten times higher). Although the incubation medium made of citrate buffer provides somewhat higher activity than that with phosphate buffer, we prefer to use phosphate in the standard procedure (Fig.4) which allows the comparison with other authors' results.

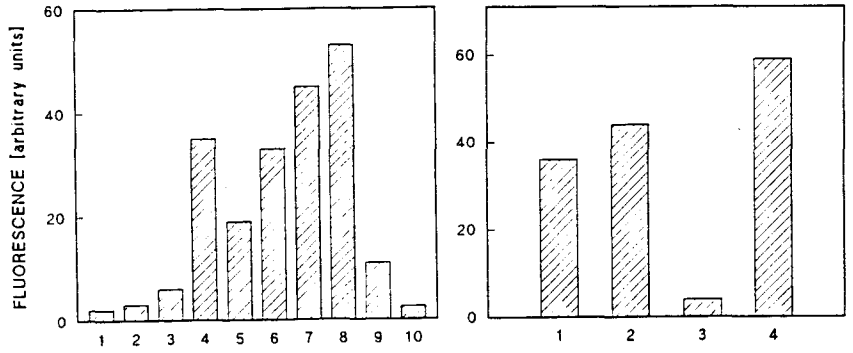

Fig.3. The enzyme activity in media with different composition (phosphate buffer, the same amounts as in the standard medium; sarkosyl 0.1 % v/v, 1 - sarkosyl, 2 - Triton, 3 - EDTA, 4 - β-mercaptoethanol, 5 - EDTA+β-mercaptoethanol, 6 - Triton+EDTA, 7 - Triton+β-mercaptoethanol, 8 - Triton+EDTA+β-mercaptoethanol, 9 - Triton+EDTA+β-mercaptoethanol+sarkosyl, 10 - plain buffer.

Fig. 4. The activity in incubation media based on different buffers (pH 6.5; 1 - acetate buffer, 2 - phosphate buffer, 3 - tris-maleate buffer, 4 - citrate buffer).

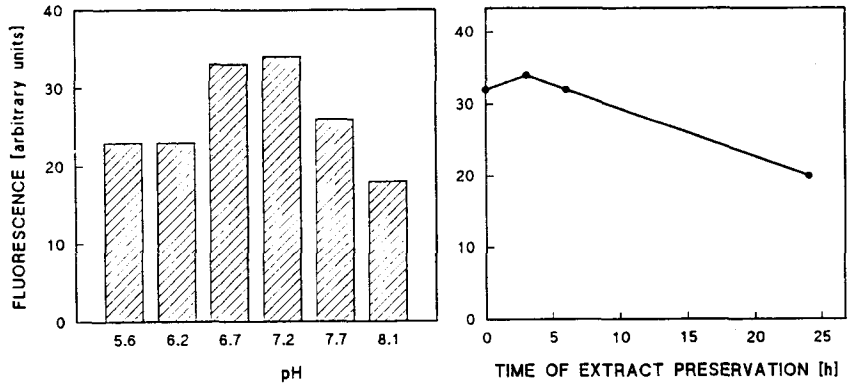

Fig. 5. The activity in the standard incubation media at different pH.

Fig. 6. The stability of the extract (*e/i* buffer, activity after the given time of preservation).

For evaluation the stability of homogenate the activity was measured using fresh homogenate and then after 3, 6 and 24 h storage in a refrigerator (8 °C) in darkness. The homogenate was fairly stable (Fig. 6). We also tested the stability of the incubation medium, assaying the activity of the same homogenate using incubation medium of different age (Fig. 7). It follows that the incubation media can be used

even after one month storage in a refrigerator in darkness. The fluorescence of final solution (after mixing with stop-solution) does not change during 24 h when kept free in the laboratory (Fig. 8). The reliability of fluorescence measurements was investigated by gradual dilution of the given sample by the stop-solution containing corresponding amount of *e/i* buffer (Fig. 9).

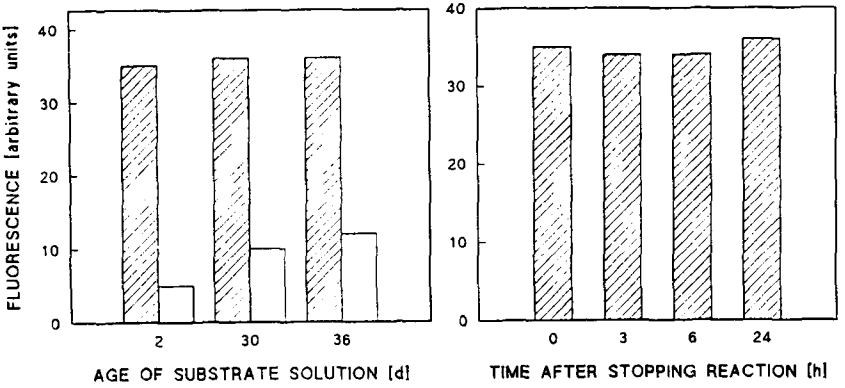

Fig. 7. The stability of the incubation medium (*hatched columns* - samples, *empty columns* - blanks).
Fig. 8. The stability of the final reaction product (the same sample measured after the given time).

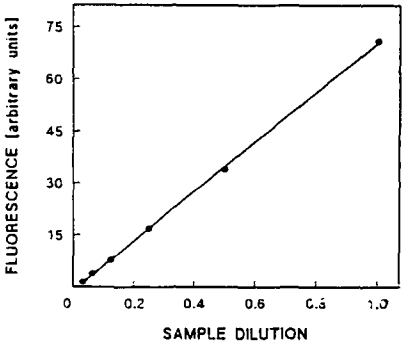

Fig. 9. The proof of the reliability of the fluorescence measurements (gradual dilutions of the sample).

The accuracy and reliability of the assay was verified in 9 paralel assays of the same material. The final fluorescence was measured repeatedly three times immediately after stopping the reaction and three times 1 h later (Table 1). Other data in this respect can be obtained from Fig. 6 and Fig. 7. Fig. 6 shows several independent assays using the same incubation medium and the same extract either fresh or stored for 3 and 6 h, as given previously. Fig. 7 represents parallel assays using the same extract and different incubation media. The variation of particular measurements is within the range $\pm$ 10 % of the mean.

When comparing method of Jefferson (Jefferson 1987, Jefferson *et al.* 1986, 1987) and its modifications (Bustos *et al.* 1989, Hu *et al.* 1990, Kosugi *et al.* 1990,

Putteril and Gardner 1989, Sporlein *et al.* 1991) our work is a contribution to efforts to find the most convenient and reliable procedure for quantitative GUS assays.

Table 1. The accuracy of the procedure tested by parallel assays and repeated measurements

Sample	Fluorescence measurement					
	1	2	3	4	5	6
1	49	50	50	51	53	51
2	53	53	54	56	56	56
3	54	53	56	56	58	57
4	54	53	56	57	58	57
5	52	52	55	56	56	56
6	53	55	57	58	58	58
7	53	54	56	57	56	57
8	54	54	56	57	57	57
9	54	53	55	57	56	56

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