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Effects of thionins on β-glucuronidase in vitro and in plant protoplasts

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Thionins cause the irreversible inactivation of β-glucuronidase (GUS) in vitro in a dose- and time-dependent manner. The enzyme is also sensitive to externally added thionins when expressed in the cytoplasmic compartment of tobacco protoplasts transformed with the *Gus* gene under the 35S promoter of the cauliflower mosaic virus. In protoplasts transformed with the *Gus* gene fused to a signal peptide, where GUS is translocated into the lumen of the endoplasmic reticulum, the activity is significantly increased both by externally-added and by transiently-expressed thionin, suggesting that it interferes with GUS secretion.

β-Glucuronidase; Thionin; Tobacco protoplast; Transient expression

1. INTRODUCTION

Thionins are cysteine-rich polypeptides of about 5 kDa that have been isolated from a variety of plant species (for reviews, see [1–3]). The toxicity of thionins to different kinds of organisms and to cells in culture has been investigated over several decades, following the initial reports of their antibiotic properties [4,5]. Toxicity to bacteria [6], yeast [4,7], fungi [2,8], animal cells [9,10] and whole animals [10–12] has been demonstrated. Current interest in these proteins relates to their possible involvement in plant defense [6]. Apart from this possible role, no specific function has been found for these proteins, although it has been suggested that they may participate in thioredoxin-mediated metabolism, based on their in vitro redox properties [13,14]. We now report the inactivation of β-glucuronidase (GUS) by thionins, both in vitro and in plant protoplasts.

2. MATERIALS AND METHODS

An equimolar mixture of α1- and β-thionins from wheat, highly purified by high-performance liquid chromatography was the gift of A. Molina (Madrid, Spain). Purified β-glucuronidase from *Escherichia coli* used in this study was supplied without added buffer by Sigma (G2871).

Transgenic tobacco plants expressing the β-glucuronidase gene [15], with and without the signal peptide from the wheat α-thionin gene (hereafter *SP-Gus* and *Gus*, respectively), under the 35S promoter from the cauliflower mosaic virus, had been obtained and checked for expression in *Nicotiana tabaccum* W38 in the course of previous work (M.J. Carmona, unpublished), as was the construction of a fusion (*35S-α1TH*) involving the same promoter, the sequence coding for α1-thionin from wheat, and the termination signals from the octopine-synthase gene (M.J. Carmona, unpublished).

Leaf protoplasts were isolated from axenic shoot cultures of transformed (*Gus* and *SP-Gus*) and non-transformed *N. tabaccum* W38 essentially following established procedures [16]. Purified protoplasts were plated at 2.5–5.0 × 10^5/ml in MSP$_1$9M medium and incubated at 27°C in the dark when indicated. Tunicamycin (Sigma) was added to the medium at 5 μg/ml to inhibit glycosylation.

Protoplasts from *SP-Gus* plants were transiently transformed with the *35S-α1TH* construction by the polyethylenglycol method [16], using 30 μg of plasmid plus 75 μg of carrier salmon sperm DNA, for 10^6 protoplasts. Protoplast's viability was monitored by staining with Evan's blue.

GUS activity was determined by the fluorometric assay [15]. Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12–20% polyacrylamide minigels from Bio-Rad according to the manufacturer's instructions and either stained for protein, by the silver nitrate procedure (Merck), or for enzyme activity, by incubation in the fluorometric assay mixture [17].

3. RESULTS

3.1. *In vitro inactivation of GUS by thionins*

Preliminary experiments concerning the possible effects of thionins on signal peptide-mediated export of GUS by plant protoplasts suggested a likely direct inactivation of this enzyme by thionins. This was confirmed by treating purified GUS from *E. coli* with a mixture of α1 and β-thionins from wheat endosperm: while the enzyme was fairly stable in the medium used for tobacco protoplasts over a 3-h period, it was inactivated in the presence of 40 μM thionin with a half-life of about 2 h (Fig. 1). Incubation of purified GUS with tobacco protoplasts also led to inactivation (Fig. 1), which might explain the erratic results sometimes encountered when using GUS as reporter enzyme in transformed plants and the low 'secretion index' previously found for GUS [18].

Purified GUS was incubated with different thionin concentrations and the reaction mixtures were subjected to SDS-PAGE. The electrophoretic gels were stained for protein and for enzyme activity (Fig. 2A). Both the

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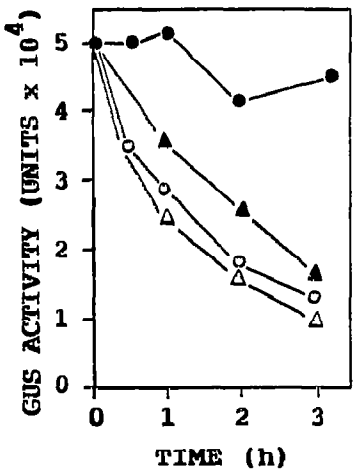

Fig. 1. Effect of thionins and protoplasts on purified bacterial β-glucuronidase (GUS). GUS activity in MSP_1 9M medium (●) or in the same medium plus the following additions: 40 μM α + β-thionin from wheat endosperm (▲), tobacco protoplasts (5×10^5/ml) (○) and thionin (40 μM) plus protoplasts (5×10^5/ml) (△). The MSP_1 9M medium is described in ref. [16]. GUS activity is expressed in relative fluorescence units.

amount of GUS protein stained and the enzyme activity detected 'in situ' significantly decreased as the thionin concentration increased, indicating that GUS was irreversibly inactivated. In a separate experiment, a concomitant decrease with time of both GUS protein and thionin was observed (Fig. 2B). No new band appeared in the gel as a result of the interaction of GUS with thionin.

3.2. *Effects of thionins on GUS activity in protoplasts*

Protoplasts from transformed *Gus* tobacco plants had high levels of GUS activity, whereas those from *SP-Gus* plants did so only after overnight incubation with the glycosylation inhibitor tunicamycin (5 μg/ml), which is in line with previous observations [19]. Both types of protoplasts were treated with tunicamycin for 22 h and then with thionin (Fig. 3). The extracellular to intracellular (*E/I*) ratio of steady-state GUS levels was greater for the *SP-Gus* than for the *Gus* protoplasts. The effect of externally added thionin on intracellular GUS activity was markedly different in the two types of protoplasts: while it rapidly decreased upon addition of thionin in *Gus* protoplasts, it significantly increased within protoplasts with the *SP-Gus* fusion (Fig. 3). Extracellular GUS was significantly affected in both cases (Fig. 3). Protoplasts were sensitive to added thionin (20 μM) with a half-life of about 20 h.

Protoplasts from *SP-Gus* plants were transiently transformed in the presence of polyethylenglycol [16] with the *35S-α1TH* construction encoding the α1-thionin from wheat. When tobacco is stably transformed with this construction, mature thionin is produced after processing of a higher molecular weight precursor (unpublished). The same plasmid without the coding sequences was used as a control. After incubation for 3 h, tunicamycin (5 μg/ml) was added and the protoplasts were further incubated for 22 h. Transient expression of the thionin gene led to a significant increase in the GUS accumulated within the protoplasts as compared with the control (Fig. 4A), while protoplast viability was not differentially affected (Fig. 4B). No GUS activity was detected in the supernatant, probably due to increased extracellular proteolysis resulting from the transformation treatment.

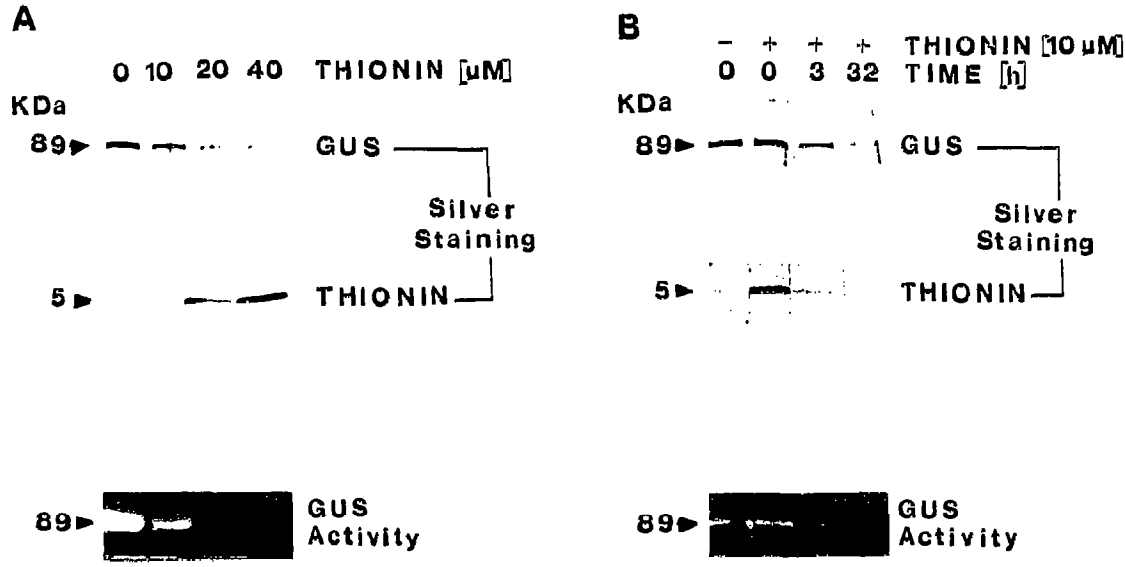

Fig. 2. Inactivation of GUS by thionins (α +β). (A) GUS (7.5 units/track) was incubated in the presence of the indicated amounts of thionins for 3 h and then subjected to SDS-PAGE for 1 h, silver stained for protein or incubated with the fluorometric assay mixture to stain for enzyme activity [17]. (B) Time course of the reaction between GUS (5.0 units/track) and thionin (10 μM).

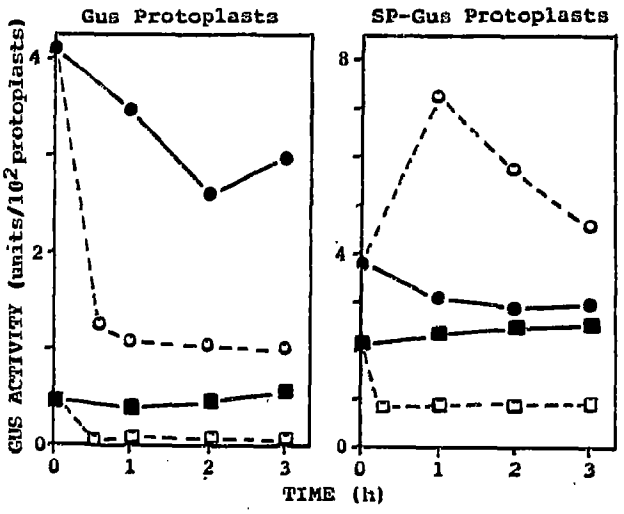

Fig. 3. Effects of externally-added thionins on GUS activity in protoplasts. After tunicamycin treatment (5 μg/ml, 22 h), *SP-Gus* and *Gus* protoplasts were incubated with (○,□) and without (●,■) 40 μM thionin. GUS activity was determined in the cell fraction (○,●) and in the medium (□,■). Activity is expressed in relative fluorescence units per 100 protoplasts.

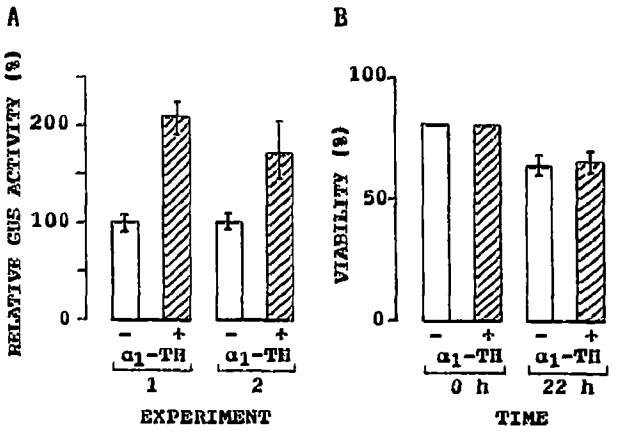

Fig. 4. Effect of transiently expressed α1-thionin (α1-TH) from wheat endosperm on *SP-Gus* tobacco protoplasts. (A) Effect on GUS activity within protoplasts. (B) Protoplast's viability estimated with Evan's blue.

4. DISCUSSION

Although activation of fructose biphosphatase (FBPase) by reduced thionin with or without thioredoxin has been shown previously [13,14], no enzyme inactivation by this protein has been reported. The present results show that purified GUS is inactivated by oxidized thionin in a process that implies the concomitant conversion of both GUS and thionin into forms that are either insoluble or do not move into the electrophoretic gel under the experimental conditions used. The possible covalent union between thionin and the enzyme through disulphide exchange merits further investigation.

The inactivation of GUS that occurs in tunicamycin-treated *Gus* protoplasts is in sharp contrast with the activity increase observed in those with the *SP-Gus* gene construction (Fig. 3). These results suggest that externally added thionin has ready access to GUS in the cytoplasmic compartment, while that translocated into the lumen of the ER is protected. In this case, it seems that the added thionin interferes with GUS secretion and that the sharp decrease in extracellular GUS activity upon thionin treatment is not only the result of direct inactivation but of reduced export. Transient expression of the nucleotide sequence coding the full thionin precursor in *SP-Gus* protoplasts leads to GUS accumulation, with no differential effect on protoplast viability. The levels of thionin produced by transient expression, which have not been determined, seem to be sufficient to interfere with GUS export but are either insufficient for significant GUS inactivation or active thionin does not come into contact with GUS, in spite of the fact that they are both initially translocated into the lumen of the ER.

The approach demonstrated here with GUS as model enzyme is a first step towards the study of the possible effects of thionins on relevant plant enzymes.

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