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# Activity of Maize Transglutaminase Overexpressed in *Escherichia coli* Inclusion Bodies: An Alternative to Protein Refolding

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*Transglutaminases (TGases) catalyze protein post-translational modification by ε-(γ-glutamyl) links and covalent polyamine conjugation. In plants, this enzyme is poorly characterized and only the maize plastidial TGase gene (*tgz*) has been cloned. The* tgz *gene (Patent WWO03102128) had been subcloned and overexpressed in Escherichia coli cells, and the recombinant protein (TGZp) was present mainly in inclusion bodies (IB) fraction. In this work, after overexpression of TGZ15p and SDS-PAGE IB fraction analysis, bands about 65 and 56 kDa were obtained. Western blot, alkylation and MALDI-TOF/TOF analyses indicated that the 56 kDa band corresponded to a truncated sequence from the native TGZ15p (expected MW 65 kDa), by elimination of a chloroplast signal peptide fragment during expression processing. So that large-scale protein production and protein crystallization can be applied, we characterized the TGZ15p enzyme activity in the IB protein fraction, with and without refolding. Results indicate that it presented the biochemical characteristics of other described TGases, showing a certain plant-substrate preference. Solubilization of the IB fraction with Triton X-100 as nondenaturing detergent yielded active TGZ without the need for refolding, giving activity values comparable to those of the refolded protein, indicating that this is a valuable, faster way to obtain TGZ active protein.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 27: 232–240, 2011
*Keywords:* E. coli *protein overexpression, inclusion bodies fraction, enzymatic activity, nondenaturant solubilization, maize transglutaminase*

## Introduction

Transglutaminases (glutamine:amine γ-glutamyl-transferase, E.C.2.3.2.13) (TGases) are enzymes that catalyze posttranslational modification of proteins by inter- or intramolecular cross-linking through acyl-transfer between γ-carboxyamide groups of glutamine residues and ε-amino groups of lysines.[1] The results of this activity are the modification of the protein conformation and other more extensive conformation changes due to bonding between the same protein and between different proteins, forming high molecular weight conjugates. The interest in these enzymes is focussed on clinical applications (they are implicated in neurodegenerative diseases, blood coagulation, etc.), food additives (texturing agents), wool textiles, and biopolymers.

TGases and their functionality have been less studied in plants than in humans and animals. In previous studies, we

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immunolocalized a maize plastidial TGase mainly in the grana-appressed thylakoids of mesophyll light-exposed cell chloroplasts.[2] In assays with plant extracts (isolated maize chloroplasts, thylakoids, and grana), it has been showed that EGTA inhibits the TGase activity of maize chloroplast extracts without exogenous calcium.[3] Blocking compounds such as diethyldithiocarbamic acid, dithiothreitol (DTT), GTP, and Diuron also inhibited maize TGase activity. Plant TGase activity has been found to be significantly higher when the enzymatic assay is performed in the light.[4] The first cloning of plant TGases, by our group, revealed two cDNAs that code for chloroplast maize TGases, termed TGZ15 and TGZ21 according to the number of repeats found in the noncatalytic enzyme domain.[4,5] These maize cDNA clones exhibit TGase activity and the general biochemical characteristics described for nonplant TGases. By overexpression of TGZ protein in *E. coli*, we have also demonstrated that the protein is mainly produced in the inclusion body (IB) fraction, and consequently, a refolding process was necessary to obtain active protein.[6,7]

In the overexpression of heterologous genes, a suitable protein production process is critical to establish an effective expression and purification system for any target protein. *E. coli* has been widely used as a host for the production of recombinant proteins as it is the best characterized bacterium.[8] The fate of any recombinant protein within *E. coli* is typically one of the following: proper folding to a native biologically active form, proteolytic degradation, or accumulation in the form of IBs. The characteristics of the protein to be produced usually dictate the choice of expression system and/or conditions to be explored first. Obtaining overexpressed protein from IBs has several advantages, including simple protein recovery, less contamination by endogenous proteins, and less degradation by proteases.

Attempts at in vitro folding of IB proteins has proved difficult and, as with protein purification, protein folding protocols have to be developed on a case-by-case basis. Various procedures for in vitro IB protein folding are available, and some proteins used for functional or structural analysis, or for industrial applications, are routinely produced by refolding of solubilized IB fractions.[9] Ideally, a folding protocol should allow complete recovery of proteins from IBs, but yields of ~30% are considered a success. However, for proteins such as mammalian subtilisins, N-terminal propeptides act as intramolecular chaperones, essential for native structure formation of more C-terminal domains.[10–12] In these cases, no refolding of recombinant products is possible if large parts of the propeptide are missing. Protein misfolding is frequent during bacterial overexpression of recombinant genes.[13] Recent studies on protein activity in bacterial IBs[14,15] indicate that protein aggregation in IBs does not necessarily imply loss of biological activity. In this case, the IBs are not mere deposits of inert protein molecules, but reservoirs of aggregated proteins that, to a certain extent, can be recovered, in vivo, as a soluble protein fraction.[16,17] This means that large amounts of pure proteins, normally expressed in the IB fraction, can be obtained, without requiring refolding being be active. This is precisely the case for TGZ4p, as shown in this article.

With respect to TGase heterologous production, only TGase from *Streptoverticillium sp*[18] is currently commercially available, with only low production by overexpression in *E. coli*.[19] Overexpression of maize TGZ protein in *E. coli* has been previously reported by our group, demonstrating that it is mainly produced in the IB fraction.[6,7] However, for large-scale protein production, as for protein crystallization, complete characterization and evaluation of the IB TGZ fraction to obtain active protein become necessary. In this article, starting from the *E. coli* protein insoluble fraction (IB), we analyzed the biochemical and enzymatic parameters of TGZ overexpression, especially in relation to its TGase specific activity. The enzymatic activity characteristics of the IB refolded protein with respect to that of IB nondenaturing protein solubilization were evaluated. The results showed that unfolding/refolding are not indispensable for reconstituting the overexpressed protein in its active form.

## Material and Methods

### *Production of recombinant protein and IB fraction*

DH5α and BL21 (DE3) *E. coli* strains were used as cloning hosts for construction of expression plasmids and for protein expression, respectively. The pBluescript and pTZ57R/T plasmids were used as cloning vectors, and pET28 as the expression vector. Cells were grown in Luria-Bertani (LB) medium supplemented with 30 $\mu$g kanamycin ml$^{-1}$ at 37°C, unless otherwise indicated. The expression plasmids were constructed as previously reported.[6] Briefly, the maize transglutaminase cDNA (*tgz15*) initially cloned in pBluescript was subcloned into the pET28-b (+) vector using the *Eco*RI and *Xho*I restriction sites. For maize transglutaminase protein expression (TGZp), transformed (pET28-*tgz*) *E. coli* BL21 colonies were grown at 37°C in 250-mL Erlenmeyer flasks with LB medium containing 30 $\mu$g kanamycin mL$^{-1}$ to an $OD_{600}$ of 0.4, induced for 5 h with 50 $\mu$M IPTG, and finally harvested by centrifugation and freezing at −80°C. Intracellular recombinant proteins were released with the B-PER® reagent (Pierce), centrifuged, the soluble fraction separated and the TGZp IB fraction resuspended in 10 mL L$^{-1}$ B-PER, following the manufacturer's instructions. Alternatively, the IB fraction was resuspended O/N at 4°C and shaken at 4 rpm in Tris-HCl 20 mM buffer adding different detergents and concentrations. After centrifugation, the supernatant was used for the activity experiments.

### *Refolding of TGZp*

Aliquots of 0.1–1mg mL$^{-1}$ of TGZp from the IB fraction, purified as described previously,[6] were incubated in B9 refolding buffer (Fold Screening kit, Hampton Research, Foldt™ HR2–229) (55 mM MES, 264 mM NaCl, 11 mM KCl, 0.055% [w/v] PEG 3350, 550 mM guanidine-HCl, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, and 440 mM sucrose) for 6 h at 4°C with agitation (4 rpm). Solutions were dialyzed against the recovering buffer (55 mM Mes, 50 mM NaCl, 1 mM DTT, 1.5 mM $Ca^{2+}$, 50 mM L-Arg/L-Glu, and 0.5% B-O) to remove the B9 buffer and precipitate the unfolded proteins, then analyzed for correct protein folding using the TGase-activity assays described below. A 30 mL refolding assay was performed using B9 buffer and 0.1 mg mL$^{-1}$ TGZ4p.

### *Transglutaminase activity determination*

*Radioactive Method.* This method is based in the incorporation of 1.4 (*n*)-$^3$H]-putrescine (Put; specific activity 962 GBq/mmol)[3] into thylakoid proteins or *N*,*N*-dimethylcasein (DMC), using a folded TGZp IB fraction or a resuspended TGZp IB protein fraction, after repeated precipitations. The

radioactivity was measured in a scintillation counter (Beckman LS 6000 SC, Fullerton, CA), and the activity was expressed in pmols of transformed Put per mg protein per hour. For the light-assays, a white-light intensity of 90–100 $\mu$mol $m^{-2}$ $s^{-1}$ was used.

The colorimetric method is based on the procedure of Grossowicz et al.,[20] using the hydroxamate procedure with the specific TGase-substrate *N*-carbobenzoxy-L-glutaminyl-glycin (CBZ-Cln-Gly-OH). The standard reaction was for 10 min at 30°C. The calibration curve was prepared using L-glutamic acid-$\gamma$-monohydroxamate. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 $\mu$mole of hydroxamate per minute.

### *Protease activity*

The putative protease activity of TGZp was measured by the resorufin-labeled casein method (Roche), using 100 $\mu$L of B9 buffer-refolded protein solution (0.1 mg/mL) and 50 $\mu$L of substrate solution (0.4% labeled casein).

### *SDS-PAGE and Western blotting*

TGZp overexpressed proteins were analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue, following the Laemmli method, or electroblotted onto a nitrocellulose membrane, incubated with the anti-T7.tag antibody (Novagen; 1:10,000 dilution) or the anti-TGZp antibody[6] (1:5,000 dilution) and both antibodies detected with anti-mouse IgG-alkaline phosphatase conjugate. The anti-T7.tag antibody recognizes a peptide with 11 amino acids encoded in the leader sequence of T7 bacteriophage gene10 and internal T7 tagged fusion proteins. In the pET28 vector, this sequence is situated before the His-Tag sequence added to the overexpressed protein. The anti-TGZp antibody was produced from *E. coli* overexpressed TGZp semipurified protein.[6] Approximately 30 $\mu$g total protein was added in each well. Bound antibodies were visualized using an ECL chemiluminescence system (Amersham Biosciences). Protein concentrations were determined with a colorimetric protein standard assay (Bio-Rad) using bovine serum albumin as the standard.

### *Reduction-alkylation*

For sample reduction, 10 $\mu$L DTT (20 mM) and $NH_4HCO_3$ (100 mM) were added to 10 $\mu$L of the TGZ IB fraction (30 $\mu$g total protein). After incubation at 56°C for 45 min, 10 $\mu$L of 110 mM iodoacetamide and 100 mM $NH_4HCO_3$ were added, and the mixture incubated at room temperature for 30 min in the dark (alkylation). Negative and positive controls were also subjected to the same conditions. Finally, 20 $\mu$L of each sample (including sample buffer) was run on SDS-PAGE.

### *MALDI-TOF analysis*

Proteins were identified by MALDI-TOF/TOF (model 4700 from Proteomics Analyzer ABI) and/or by CapLC-Q-TOF liquid-mass chromatography (Waters Micromass). Protein samples isolated from Coomassie-stained 1D gels were distained, reduced, alkylated, and finally digested with sequencing-grade modified trypsin.[21] Criteria used to accept the identification were significant homology scores in Mascot, a minimum of four peptides matched and protein sequence coverage greater than 10%.

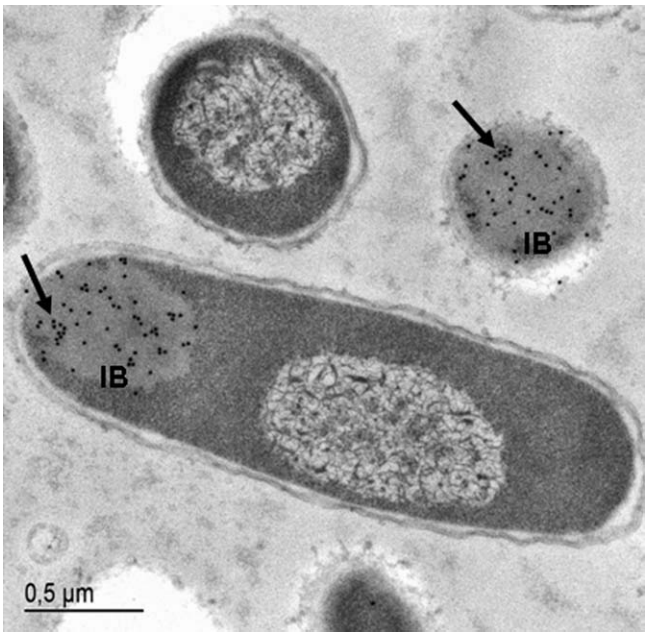



**Figure 1. Subcellular TEM-immunolocalization of TGZp overexpression in the inclusion body (IB) of *E. coli* cells.**

Arrows indicate TGZ protein detected using AbTGZ (1:3000), obtained from purified TGZ4p.[7] Samples were incubated with the secondary antibody conjugated with 15 nm gold particles. Observations were performed with a TEM Jeol JEM 1010 at 80 kV. Arrows indicate TGZp presence. IB = inclusion body.

### *Polymerization assay*

DMC polymerization by the TGZp IB fraction was tested by incubation of 3 mg $ml^{-1}$ total protein enzyme fraction with 8 mg $mL^{-1}$ DMC diluted in 20 mM Tris-HCl at pH 6.5 and 8, respectively, for 16 h. Ten microlitres of assayed sample was separated by 10% (w/v) SDS-PAGE using a buffer sample without DTT, and the bands quantified by densitometry.

## Results

### *TGZp accumulation in the IB fraction*

As previously found,[6,7] the AbTGZ polyclonal antibody obtained from purified TGZp localized the TGZp protein in a unique IB of the transformed *E. coli* cells (Figure 1). These samples were used in the following experiments.

*Reduction-Alkylation.* Analysis of the TGZ IB fraction by SDS-PAGE always gave principally two TGZ bands, one of ~65 and one of 56 kDa. As the expected MW for the complete expressed protein is 65 kDa (including the poli-His, the T7-tag sequence and a part of the vector sequence previous to the TG15 protein) (see Table 1), the 56 kDa band might be a result of aggregation between monomers of this 65 kDa form that produced a different and more rapid migration in the SDS gel. Reduction and alkylation experiments were carried out to clarify whether one of the two cysteines in the protein sequence[4,5] was responsible for possible disulfide links generating these aggregates. The results of SDS-PAGE after reduction-alkylation are showed in Figure 2a (each treatment in duplicate). The lack of significant differences between treatments indicates that cysteines are not responsible for this lower molecular mass band.

*Western Blot Immunodetection.* When the specific anti TGZp antibody[6,7] was used to immunolocalize TGZp in the IB fraction, two protein bands of the same MW (about 65 and 56 kDa) (Figure 2b, samples 1 to 5) as detected in the SDS-

**Table 1. N-Terminal Sequence of TGZ15p Overexpressed in *E. coli***

| | |
|---|---|
| TGZ15c | MGSSHHHHHH SSGLVPRGSH MASMTGGQQM GRDPNSARAH |
| TGZ15t | |
| TGZ15c | 1 **MAHRGHLDGL TGQAPALMRH GSFAAGSLSS RSPLQSSTLE** 40 |
| TGZ15t | 1 **MAHRGHLDGL TGQAPALMRH GSFAAGSLSS RSPLQSSTLE** 40 |
| TGZ15c | 41 **MLENKLA** 47 |
| TGZ15t | 41 **MLENKLA** 47 |

Normal letters: His-tag and pET-28a vector sequence (4.3 kDa). Normal-underlined letters: T7-tag sequence. Bold letters: TGZ15p chloroplast signal peptide (5.2 kDa). Bold-underlined letters: Mascot search results indicating matched peptides from the MS/MS analyses after trypsin digestion. TG Z15c, complete (65 kDa) TGZ15p. TGZ15t, truncated TGZ15p (56 kDa).

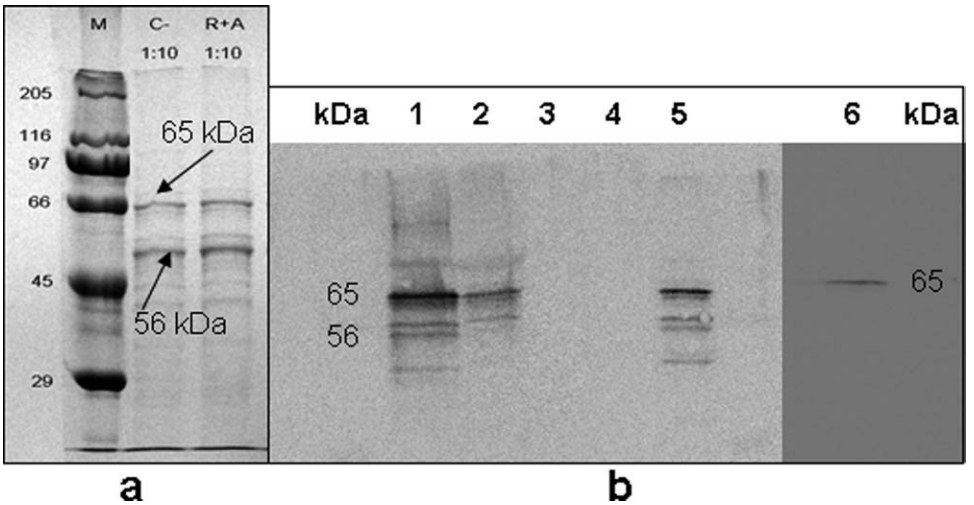
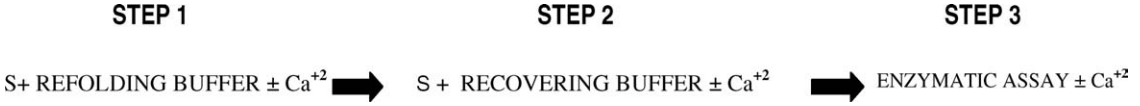



**Figure 2. a: SDS-PAGE analysis of the TGZ-IB fraction from the reduction/alkylation treatments, indicating 65 KDa and 56 KDa bands and protein concentrations. b: TGZp WB immunodetection from IB fraction using AbTGZp (1:5000) (samples 1 to 5) and AbT7.tag (Novagen) (1:10000) (sample 6). 1, IB fraction in 8 M urea; 2, IB insoluble fraction in B-PER O/N; 3 and 4, wash fractions, 5 and 6, IB soluble fraction in B-PER O/N. C-, negative control; R, reduction; A, alkylation; R+A, reduction and alkylation; M, molecular weight markers.**




**Scheme 1. Effect of calcium on TGZp refolding and on the final enzymatic assay.**

PAGE analysis were obtained, indicating that these bands correspond to the overexpressed protein. However, when the anti-T7.tag antibody was used with the same fraction, only a 65 kDa band was obtained (Figure 2b, sample 6), indicating that the 56 kDa protein band lacks minimum the aa sequence including the T7-tag sequence (4.3 kDa in total, Table 1) http://www.ncbi.nlm.nih.gov), and probably a chloroplast signal peptide fragment to complete the MW loss observed.

*MALDI-TOFF Analysis of TGZ Bands.* *MALDI-TOFF* analyses confirmed the 65 and the 56 kDa bands obtained by SDS-PAGE from the IB fraction, as TGZ proteins. The difference in the N-terminal sequence digestion indicated that, as in other overexpressed *E. coli* proteins having signal peptides, the sequence of about 56 kDa band corresponded to a truncated protein (produced by partial *E. coli* peptidase digestion) missing an important part of its signal peptide (from aa 1 to aa 30) (see Table 1). These observations confirmed the Western blot results.

### *TGZp refolding from IB fraction*

The following experiments were realized to characterize the activity of TGZp *E. coli* overexpressed, to compare the efficiency of the IB protein refolding process with the active protein IB solubilization process.

*Calcium Dependence Along the Refolding Process and pH-Stability Test.* In previous papers,[4,7] we had demonstrated that, like the majority of TGases, TGZ activity is $Ca^{2+}$dependent. To characterize this $Ca^{2+}$ dependence during the all process of protein refolding and after it, the effect of this ion on TGZ activity in each step was tested (Scheme 1; Figure 3). Using procedures already published by our group, TGZp was overexpressed in *E. coli*, the IB insoluble fraction solubilized with urea 8 M, affinity purified and refolded with B9 buffer[7] in the sequence of the following refolding process (see Scheme 1).

The highest TGZp activity was that obtained with $Ca^{+2}$ added to the refolding buffer and to the final activity assay, but not to the recovering buffer (Figure 3, 2+Ca). These results indicate that, as expected, $Ca^{2+}$ has a functional role on TGZ activity, contributing to the recovery of its functional structure during the refolding process. Using the selected $Ca^{2+}$ conditions, a stability test was carried out in the absence or presence of a stabilizing solution composed by 35% glycerol, 50 mM L-arg/L-glu, and 0.1% $\beta$-octyl–glucopyranoside (B-O), freezing the refolding samples at $-20°C$ for 7 days at pH 6.5 or 8.0 and, afterwards, measuring its TGase activity in front of fresh-refolded samples as a control. A small amount of TGZ activity (25% of the control samples) was maintained only in

the presence of stabilizing solution at pH 6.5, indicating that protein stability was very low. The optimum calcium and pH conditions were used for all the following experiments.

*Light Dependence and Substrate Preference*. In previous papers, the in vivo TGase light activation using different plant protein extracts has been demonstrated.[2,4,22] To test the activity of recombinant refolded TGZp (rTGZ) over its natural substrates, two experiments were realized. In a first experiment, the endogenous TGase activity of light-grown plants thylakoid extracts in the presence or in the absence of rTGZ was measured, resulting that TGase activity was 2.6-fold higher when rTGZ was present (Figure 4a). To analyze the effect of light over refolded TGZp activity, maize thylakoid protein extracts from light and dark grown plants were used as substrates for refolded TGZp, and its activity was measured. Figure 4b shows that the incorporation of $^{3}$H-Put by the recombinant protein were 100-fold greater using light-grown than dark-grown thylakoid protein extracts. However, if DMC was used as TGZp substrate, the obtained activity was not light dependent, and significantly lower than that of light-grown plant substrates. In summary: (i) TGZp activity is higher in the presence of its natural plant substrates than in the presence of DMC and (ii) TGZp activity is light dependent only when plant substrates were used.

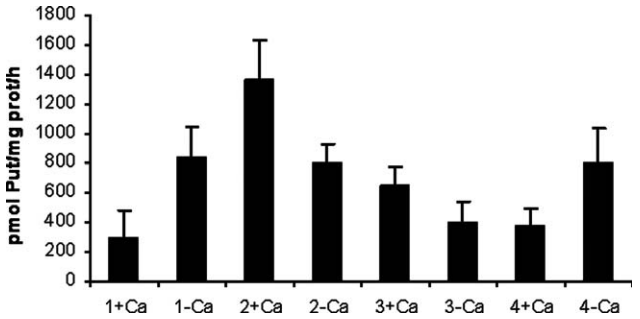



**Figure 3. Effect of $Ca^{2+}$ on TGase activity (pmols Put/mg prot.h) of overexpressed TGZp during the refolding process. 1 = $Ca^{2+}$ in refolding and in recovering buffers; 2 = $Ca^{2+}$ in refolding and not in recovering buffers; 3 = $Ca^{2+}$ presence only in recovering buffer; 4 = $Ca^{2+}$ absence in refolding and in recovering buffers. +Ca = $Ca^{2+}$ presence in the activity assay; –Ca = $Ca^{2+}$ absence in the enzymatic assay.**

Refolding buffer contained 2.2 mM $CaCl_2$. Recovering buffer contained 1.5 mM $CaCl_2$. Enzymatic assay contains 5 mM $CaCl_2$.

*Protease Activity*. Owing to the putative protease characteristics described for some TGases, protease activity assays at pH 5, 6, 7, and 8 were performed with the refolded TGZp-IB fraction. The results showed that, at these pH values, refolded TGZp did not have protease activity (data not presented).

### *Activity of TGZp IB fraction resuspended in nondenaturing detergents*

To determine the existence of TGase activity in the resuspended IB-TGZp fraction without needing a long and expensive refolding process, a comparative activity experiment measuring the $\mu$mols of transformed substrate per mg of total protein per min, using refolded and nonrefolded TGZp-IB fractions was carried out (Figure 5). In the standard procedures, after centrifugation, soluble protein fraction is separated from insoluble IB fraction (apparently containing inactive proteins) which is dissolved in urea and after submitted to the refolding process. As can be seen in Figure 5, unexpectedly, the highest TGase activity was detected in the IB fraction when it was resuspended in B-PER buffer (described as a very useful IB resuspension buffer to use before the refolding process), instead of urea. This obtained activity was comparable to that of the IB TGZp fraction refolded in B9 buffer (IB + B9) and dialyzed against the

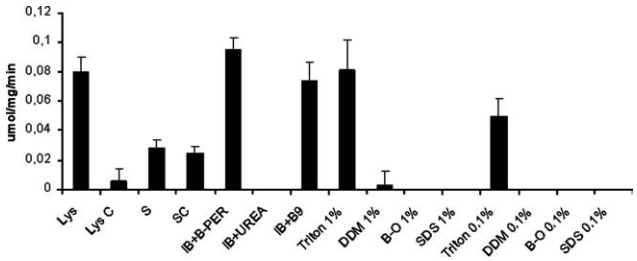



**Figure 5. TGase activity ($\mu$mol substrate/mg total protein.min) measured with the colorimetric method (see Material and Methods) in the different TGZ-over expressing *E. coli* cellular fractions, solubilization buffers and detergents.**

Lys, total induced lysate; LysC, total noninduced lysate; S, induced soluble fraction; SC, noninduced soluble control fraction; IB + B-PER, IB fraction B-PER solubilized. IB + UREA, IB fraction solubilized with 8 M urea; IB + B9 = IB fraction refolded with B9 buffer and dialyzed against the recovering buffer described in Material and Methods. Triton, Triton X-100; DDM, *n*-Dodecyl-$\beta$-D-Maltoside; B-O, $\beta$-octyl–glucopyranoside; SDS, sodium duodecylsulphate.

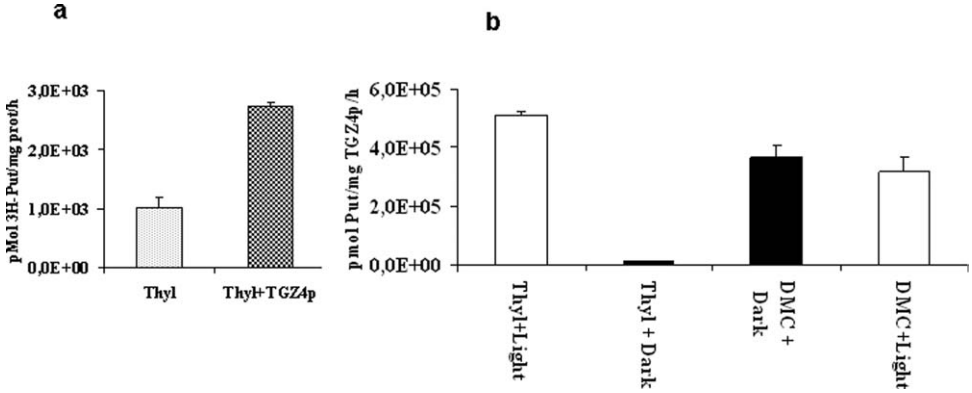



**Figure 4. TGase activity of refolded TGZp after the addition of 25 mg/mL maize protein thylakoid extracts (Thyl) or 4.6 mg/mL DMC to the enzyme assay.**

a: $^{3}$H-Putrescine incorporation per mg of total protein in presence of 100 $\mu$g TGZp/mL. b: $^{3}$H-Putrescine incorporation per mg of TGZp. DMC = dimethyl casein; Thyl = maize thylakoid extract; Light = lamp illumination assay; Dark = dark conditions assay (see Material and Methods).

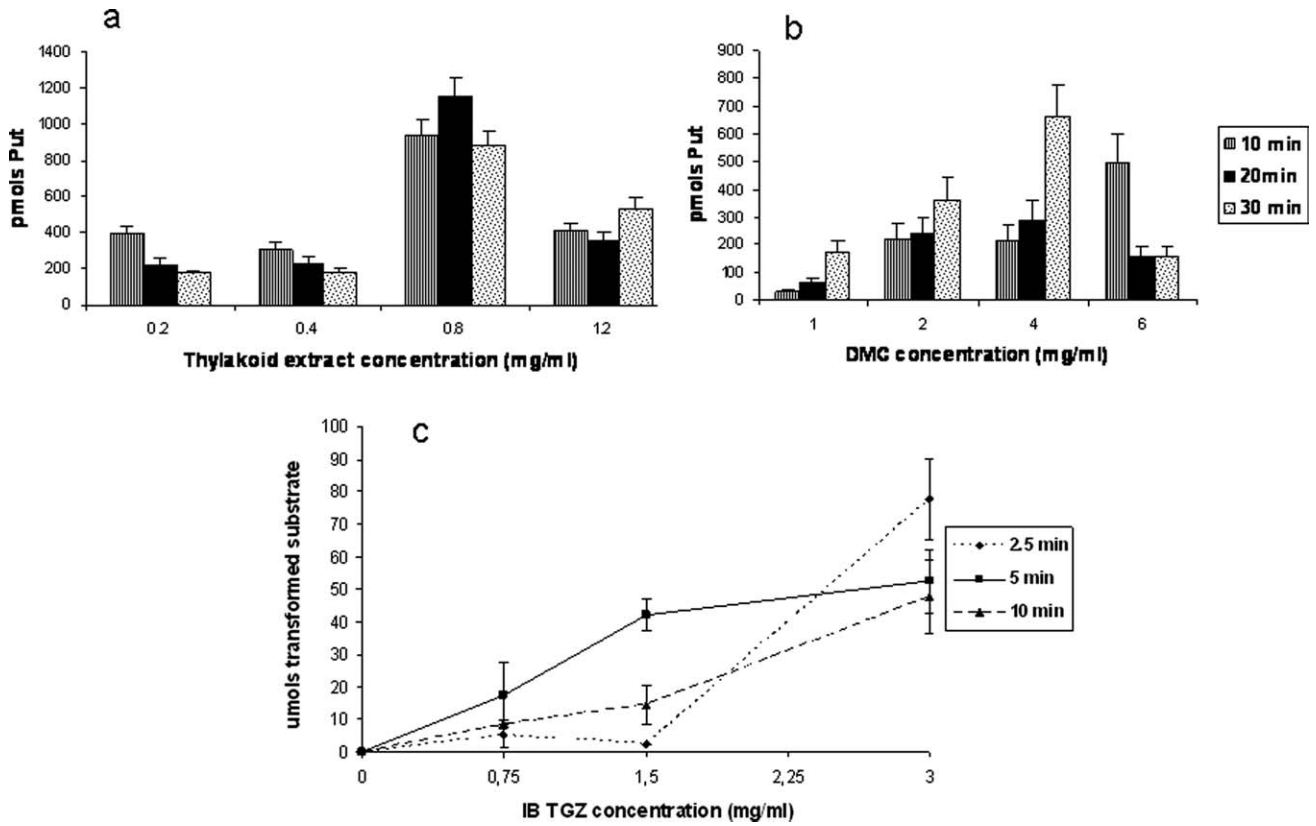



**Figure 6. a, b: TGase activity (pmols Put/mg prot.h), measured with the radioactive method, of TGZ IB fraction solubilized with 1% Triton + 35% Glicerol.**

a: Using as substrate 0.2, 0.4, 0.8, and 1.2 mg of maize thylakoid protein extract/mL, respectively, after 30, 60, and 90 min of assay reaction. b: Using as substrate 1, 2, 4, and 6 mg DMC/mL, respectively, after 10, 20, and 30 min of assay reaction. c: Substrate transformation velocity ($\mu$mol transformed substrate) of four different concentrations of TGZ15p-IB fraction (mg/mL) and three reaction times (2.5, 5, and 10 min), using 0.05 M CBZ-Cln-Gly-OH as substrate (hidroxamate method).

**Table 2. TGase Activity ($\mu$mol Transformed Substrate) of TGZ IB Fraction Solubilized with 1% Triton + 35% Glicerol, Using as Substrate 10, 30, 50, and 100 mM of CBZ-Cln-Gly-OH, Respectively (hidroxamate method), After 10, 20, and 30 min of Assay Reaction**

| Substrate Conc (mM) | 10 | 30 | 50 | 100 |
|---|---|---|---|---|
| 10 min | 0.2016 ± 0.06 | 0.3123 ± 0.08 | 0.7762 ± 0.02 | 0.3022 ± 0.07 |
| 20 min | 0.2250 ± 0.07 | 0.3670 ± 0.07 | 1.0941 ± 0.08 | 0.2873 ± 0.07 |
| 30 min | 0.0491 ± 0.002 | 0.1507 ± 0.05 | 0.1194 ± 0.03 | 0.1507 ± 0.03 |

recovering buffer, as described in Material and Methods. Control samples as LysC (total noninduced lysate) or SC (noninduced soluble fraction) are not significantly different between them and presented only a basal TGase activity. The urea resuspended IB fraction (IB + urea) do not presented any TGase activity. All these data indicate that the measured TGase activities of the induced TGZp-containing samples (IB+B-PER and IB+B9) corresponded to that of TGZp. In a further experiment, commercial B-PER buffer was substituted by nondenaturing detergents to test the activity of the solubilized TGZ-IB fraction. The detergents used were: SDS, Triton X-100, *n*-Dodecyl-$\beta$-D-Maltoside (DDM) and $\beta$-octyl-glucopyranoside (B-O), at a concentration of 0.1 and 1%, using 8 M urea as negative control. The results indicated that 1%Triton X-100 was the best detergent that solubilizes active TGZp from the IB fraction, with the activity being similar to that obtained with B-PER (most expensive than the other detergents) solubilized fractions (Figure 5).

### *TGZp activity characterization from the TGZp-IB fraction Triton-solubilized*

To characterize the activity of TGZp-containing IB fraction solubilized with 1% Triton, activity experiments using different TGZp substrates, concentrations, and reaction times were carried out (Figure 6, Tables 2 and 3). The $Ca^{2+}$ and pH conditions were always that selected in the refolding experiment. The assayed substrates were: maize protein thylakoid extract (as its original plant-substrate), DMC (used in many referenced experiments), and CBZ-Cln-Gly-OH® (a specific TGase synthetic substrate).

**Table 3. Substrate Affinity of TGZp IB-Triton Fraction Measured by Substrate Saturation Concentration (pmols)**

| Substrate | pmols |
|---|---|
| Thyl | 25800 |
| DMC | 16000 |
| CBZ-Gln-Gly-OH | 124000 |

*TGZp Activity Using Different Substrates.* In the case of thylakoid protein extract, radioactive uptake of tritiated putrescine (see Material and Methods) was used to measure TGase activity. The highest activity was that obtained with 800 $\mu$g protein $mL^{-1}$ and 60 min of assay reaction (Figure 6a). Above this protein concentration, the enzymatic activity decreased in all the reaction times assayed, indicating the saturation by this substrate concentration of the enzyme active centres present in the reaction mixture. Using the same method to measure the enzymatic activity, when DMC was used as

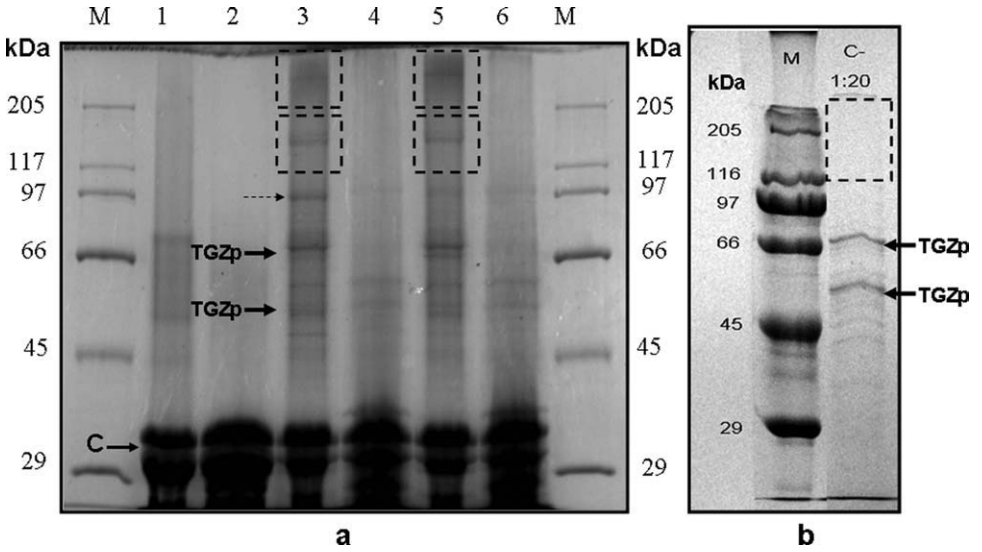



**Figure 7. DMC cross-linking by TGZp Triton-resuspended IB fraction. a: SDS-PAGE of samples after 16 h incubation with 8 mg $mL^{-1}$ of DMC in the reaction assay.**

1, DMC sample without DTT in the sample buffer; 2, as 1 with 10 mM DTT in the sample buffer; 3, TGZp fraction incubated with DMC at pH 6.5 and without DTT in the sample buffer; 4, as in 3 with DTT in the sample buffer; 5, TGZp fraction incubated with DMC at pH 8 and without DTT in the sample buffer, 6, as in 5 with DTT in the sample buffer. Discontinuous rectangles indicate high molecular weight bands. Discontinuous arrow indicates possible DMC trimer. C, casein monomer bands. b: SDS-PAGE of TGZ-IB initial samples (C-) incubated without DMC. 4. Dash rectangles, absence of high molecular weight bands. M, molecular weight markers. TGZp, TGZp bands.

substrate (Figure 6b), the saturating substrate concentration was 4 mg $mL^{-1}$ at the highest reaction time (30 min), but maximum activity was significantly lower than in the case of thylakoid extracts. As described in Material and Methods, the TGase-specific substrate *CBZ-Cln-Gly-OH* has been described as being useful to measure TGase activity of different types of samples by a colorimetric method. In this experiment, the highest activity was that obtained with 0.05 M substrate concentration. From the three different reaction times assayed, the maximal activity in all cases was after 10 min (Table 2).

*TGZp Substrate Preference and Transformation Velocity.* From the activity results, the substrate affinity of TGZp measured in terms of substrate saturation concentration (pmols) was compared (Table 3). Using the same methodology for measuring TGase activity, it can be deduced that protein thylakoid extract exhibit a higher affinity for TGZp than DMC. In the case of the hydroxamate method, the affinity of TGZp for the specific TGase-synthetic substrate was comparable to other cases referenced in the literature.[20] Attending the methodology facilities and to study the effect of enzyme concentration on the substrate transformation velocity ($\mu$mol transformed substrate), an activity experiment using the CBZ-Cln-Gly-OH substrate saturation concentration (0,05 M), three different TGZp-IB-Triton concentrations and three reaction times, was realized (Figure 6c). As the maximum activity previously found was 10 min (Table 3), the reaction times tested were 2.5, 5.0, and 10 min. As expected, the substrate transformation velocity increased with TGZp concentration in the enzymatic assay, the highest activity corresponding always to the highest TGZp concentration at the three assayed reaction times.

*Cross-Linking Activity.* To analyze the ability of TGZp to polymerise a protein substrate, as described for other TGases, a long enzymatic assay (16 h, at 30°C) using DMC as substrate of the TGZp IB-Triton resuspended fraction at pH 6.5 and 8.0, was carried out (see M & M). After the reaction, high MW bands of casein aggregates from 150 to more than 205 kDa were visualized by SDS-PAGE of samples without DTT in the sample buffer (Figure 7a). Another casein band of ~90 kDa (a possible trimer fraction) was not discarded. As can be seen in Figure 7b, these bands were not present in the original IB-TGZp fraction incubated without casein. The high molecular weight bands were absent in the DTT-treated samples and in the control DMC samples.

## Discussion

### *Significance of the two TGZp bands in the IB fraction*

Results from reduction-alkylation and MS experiments show that the obtained 56 kDa band corresponded to a truncated fraction of the native overexpressed TGZp, which has an expected molecular weight of 65 kDa.[6] This agrees with the immunodetection results using the anti-T7.tag antibody, revealing only a 65 kDa band. Considering that the originally overexpressed TGZp also contains a chloroplast signal peptide that is situated immediately after the His-tag sequence of the expression vector (N-terminal region), the decrease in MW may correspond to that of the His-tag plus the signal peptide amino acid sequences, indicating that the TGZp signal peptide is processed by *E. coli* proteases in a significant part of the total TGZp overexpression in *E. coli*. Consequently, the protein IB extract contains two TGZp proteins that may exhibit TGase activity.

### *Characterization of the IB-refolded TGZp*

As an initial step to produce pure and active TGZp on a larger scale, the biochemical characteristics of the TGZ-refolded IB fraction were analyzed. Calcium dependence was nonaccumulative, indicating that the presence of calcium throughout the process of protein refolding inhibits the activity. However, as in the majority of TGases, TGZp is calcium dependant during refolding and in the activity assay, contributing to the recovery of its functional structure during the refolding process. The relative calcium dependence for TGase activity has been previously been demonstrated in intact maize thylakoid extracts.[4] Refolded TGZp has also been shown to have low stability, based on standard

procedures of using stabilizing detergents or amino acids. Freezing of the TGZp refolded fraction, including the incorporation of glycerol, was also a restriction factor to the posterior use of the protein. In contrast, active TGZp-IB fraction can be obtained, when it is required, directly by resolubilisation in nondenaturant detergents from the frozen pellet of harvested cell fractions, without a refolding process. Furthermore, some authors have suggested that the protease activity detected in certain *Streptomyces m.* extracts overexpressing TGase[23] and in thylakoid extracts[24] might be due to a protease activity of TGases present in the extracts. In our case, purified and refolded TGZp had no protease activity, indicating that the activity measured by these authors might have been due to an independent protease included in the nonpurified extract containing the TGase enzyme.

### *Light dependence and preferential substrates for TGZp activity*

Using maize thylakoid protein extracts as substrate, our group has previously detected higher levels of TGase activity in light rather than in dark conditions, and a significant decrease in activity by plant TGase substrates inhibition.[4,22,25] Although it is under discussion whether the response to light could be ascribed to thylakoid TGase and/or to its substrates, it has been suggested that, using a maize LHCII protein preparation, animal TGase activity might be light sensitive.[26] Here, our results demonstrate that not only the native substrate preferences in the recombinant TGZp were maintained but also that the enzyme activity was light dependent only in the case when light-grown plant protein substrates were used. It is well known that activation of the photosystem II protein complex (where the principal TGZp substrates are present) depends on light.[27–29] This explains the higher TGase activity obtained with light-grown compared to dark-grown thylakoid extracts (Figure 4b), as well as the higher TGase activity of these extracts when TGZp was added to the enzymatic assay under light conditions (Figure 4a). However, if DMC (not susceptible of light-activation) is used as substrate, light-dependent activity of TGZp was not detected (Figure 4b). These results indicate that, in the case of TGZp, the substrate and not the enzyme is dependent on light activation.

### *Activity of TGZp IB fraction resuspended in nondenaturing detergents*

Protein refolding from an insoluble protein fraction is a tedious and not very productive process with respect to protein yield, limiting the number of experiments especially in the case of protein characterization and crystallization. It has been reported that active IBs can be used in suspension as efficient catalysts for bioprocesses. The activity of *Clostridium thermocellum* endoglucanase D, overproduced in *E. coli* and embedded in IBs, was close to that of the purified protein, indicating that the formation of IBs does not involve denaturation of the catalytic domain, but, more likely, the participation of this conserved region in the intermolecular reactions.[30] IBs formed by enzymes appear to be immediately useful and they can skip a refolding step because their porous nature permits substrate processing by the active enzyme molecules.[31] The biological activity of the IBs formed by different mutants of the A$\beta$42-GFP protein indicates that the kinetic competition between folding and aggregation of the protein might result in highly active inclusion bodies.[32,33] In this work, the solubilization of the TGZp IB fraction with nondenaturing detergents resulted in a high production of active protein, which has permitted us to easily realize the characterization experiments in a more comparative manner. In our comparative assays with different detergents and additives, Triton X-100 was the cheapest and most convenient detergent. The activity values obtained with the TGZp-Triton resuspended IB fraction were comparable with those obtained with the refolded protein, indicating that this is a valuable and more practical methodology to obtain TGZp active protein. The protein cross-linking results obtained once more confirm these observations (Figure 7).

### *Substrate preference of TGZp from the Triton-solubilized IB fraction*

As demonstrated in the Results, the TGZp-containing Triton-solubilised IB fraction had the same substrate preferences as the refolded protein fraction, with the thylakoid extracts the preferential substrate over DMC. These results, including the light-activity/substrate dependence, agree with the plant origin of the overexpressed protein and the enzymatic characteristics previously described using maize plants.[4] The results obtained using *N*-carbobenzoxy (CBZ)-L-glutaminylglycin, a TGase-specific synthetic substrate, with the IBs-TGZ resuspended fraction give very interesting data, indicating that TGZ exhibits similar or comparative enzymatic characteristics with respect to other TGases of nonplant origin, taking into account that this activity determination method was established for TGases of very distinct sources.[20]

## Conclusions

The characterization of TGZp overexpressed in the IB *E. coli* protein fraction presented here demonstrates that the IB fraction is valuable for the production and purification of this enzyme, and it opens the way to a new system of producing active protein without needing the tedious, aggressive and expensive unfolding/refolding steps to reconstitute the overexpressed protein in its active form. Our data also confirms that TGZp maintains the characteristics from its plant origin, with respect to substrate preferences and activity conditions, indicating that heterologous overexpression does not induce major changes in its own molecular configuration, while its enzymatic properties are comparable to that of TGases of nonplant origin. All these data point to the reproducibility and applicability of the recombinant maize TGase for use in bioprocess methodologies. Further experiments in our laboratory on TGZ purification and structural characterization are in progress.

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