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ORIGINAL RESEARCH PAPER

Purification and in vitro refolding of maize chloroplast transglutaminase over-expressed in *Escherichia coli*

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Abstract In contrast to mammalian transglutaminases (TGs), plant members of the superfamily are poorly characterized. In order to produce pure and active TG for its functional and structural studies, variants of maize chloroplast transglutaminase (TGZ, Patent WWO03102128) were sub-cloned into a pET28 vector and overexpressed in *Escherichia coli* BL21 (DE3) cells. The recombinant proteins were present mainly as insoluble inclusion bodies. The TGZ4p variant with four B-type repeats ($M_r \sim 55$ kDa), was affinity purified from urea-solubilized inclusion bodies. TGZ4p was refolded by rapid dilution in a Ca^{2+}- and guanidine-containing buffer. Active TGZ4p shows the general catalytic characteristics described for other TGs.

Keywords *Escherichia coli* · Maize chloroplast · Purification · Refolding · Transglutaminase

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Introduction

Transglutaminases (TGs, EC 2.3.2.13) catalyze post-translational protein modifications through acyl-transfer between the γ-carboxyamide groups of glutamine residues and ε-amino groups of lysines or polyamines. Animal transglutaminases possess a catalytic triad composed by a cysteine, a histidine and an aspartate, and the reaction proceeds via an intermediate linked to the cysteine. Mammalian TGs have been extensively studied and are involved in important cellular differentiation processes, tissue stabilization and apoptosis (Yee et al. 1994; Ichinose et al. 2000; Lorand and Graham 2003). Molecular and functional studies on plant TGs are still scarce. Transglutaminase activity in plants was first observed in pea seedlings (Iceckson and Apelbaum 1987), and subsequently found in organs of both lower and higher plants. Our group has previously identified a maize chloroplast transglutaminase (Villalobos et al.

2001), and we have cloned two cDNAs coding for this enzyme, *TGZ15* and *TGZ21*, which code for active TGs and possess the catalytic Cys–His–Asp triade (Torné et al. 2002; Villalobos et al. 2004). The nomenclature of the cDNAs reflects the number of B-type repeats found in the central, non-catalytic domain of the enzyme (see Fig. 1 below for a schematic representation of TGZ21 domain structure).

In industry, TGs are widely used in food texturing (Kuraishi et al. 2001; Yokoyama et al. 2004) and wool textiles modification (Cortez et al. 2004). Several food proteins, such as casein and soy, are substrates for mammalian TGs (Ikura et al. 1980). For industrial applications, as well as for structure and function studies, large amounts of protein are required, that cannot normally be obtained from natural sources. Only two preliminary studies on recombinant plant TGs production, from maize (Villalobos et al. 2004) and *Arabidopsis thaliana* (Della Mea et al. 2004), have been reported so far. At present, only TG extracted from *Streptoverticillium* sp. is commercially available, while no active plant TG has been produced for scalable and reproducible overexpression.

To produce pure and active TGZp for further studies, several variants of maize TGZ were subcloned into a pET expression vector, and overexpressed in *E. coli* cells. We also determined the conditions for purification and refolding of TGZ4p.

Materials and methods

Production of recombinant protein

E. coli strains DH5α and BL21(DE3) were used as cloning hosts for construction of expression plasmids and for protein expression, respectively. The plasmids, pBluescript and pTZ57R/T, were used as cloning vectors, and pET28 as the expression vector. Cells were grown in Luria-Bertani (LB) medium supplemented with 30 μg kanamycin/ml at 37°C, unless otherwise indicated.

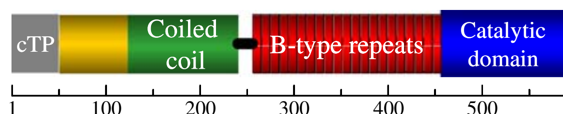

Fig. 1 Schematic representation of domain structure in maize transglutaminases (TGZ21). The depicted domain organization follows the results of ELM server (http://elm.eu.org/); ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/) predicted the chloroplast transit peptide

For the construction of expression plasmids, *TGZ15* cDNA initially cloned in pBluescript was PCR-amplified with the primers 5′-GGT CGCGGATCCGCTCATCGTGGACATCTAG-3′ (forward primer, TGZ-*Bam*HI), and 5′-CTCG AGTGCGGCCGCCCATATTTGTCTGCTC-3′ (reverse primer, TGZ-*Not*I; restriction sites are underlined). PCR was carried out using DNA polymerase high fidelity Roche, and the PCR product cloned into pTZ57R/T and sequenced. The new TGZ sequence contained four tandem repeats (TGZ4) and was subcloned into the *Bam*HI and *Not*I sites of the pET28-a (+) vector. Similar conditions were employed to generate two constructs (TGZ9 and TGZ29, lacking the predicted chloroplast signal peptide), using the 5′-GGTCGCGGATCCATG CAAACTACAGAAGTGG-3′ forward primer (Free-cTP *Bam*HI) and the above-mentioned TGZ-*Not*I primer. The original *TGZ15* sequence was also cloned into pET28-b (+) using *Eco*RI and *Xho*I restriction sites.

For protein expression, transformed (pET28-*TGZ*) *E. coli* BL21 colonies were grown in LB medium containing 30 μg kanamycin/ml to an OD_{600} of 0.4, induced for 3 h with 0.4 mM IPTG, and finally harvested by centrifugation. Intracellular recombinant proteins were released with CelLytic BII reagent (Sigma). The washed inclusion bodies (IB) were resuspended in buffer B (0.01 M Tris/HCl, 0.1 M NaH_2PO_4, 8 M urea, pH 8.0) and fractions analyzed by SDS-PAGE and/or Western blot.

Protein purification

In the case of small-scale purification trials, TGZ4p from a 200 ml culture was purified using Ni-NTA mini-spin columns under native and denaturing conditions following the QIAGEN protocol.

For FPLC Purification under denaturing conditions, cells from a 1 l culture were solubilized by gentle agitation in 10 ml buffer B for 1 h and centrifuged. The sample was applied to a 1 ml HiTrap Chelating HP column (Amersham Biosciences) connected to an ÄKTA FPLC system, and bound

TGZ4p eluted using a pH gradient (from pH 8.0 to 4.5).

SDS-PAGE and Western blotting

TGZ proteins were analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue, or electroblotted onto a nitrocellulose membrane, incubated with the anti-T7·tag antibody (Novagen; 1:10,000 dilution), and detected with anti-mouse IgG-alkaline phosphatase conjugate. 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.

Two-dimensional gel electrophoresis

Isoelectric focusing was performed in a IPGphor system (Amersham Biosciences). TGZ4p purified samples were diluted in 2% CHAPS (w/v), 8 M urea and 0.5% IPG buffer pH 3–10 (v/v) and proteins separated on 24-cm Immobiline dry strips with a linear pH gradient from 3 to 10 with a total of 50000 V/h. Before SDS-PAGE, IEF strips were equilibrated in 50 mM Tris/HCl (pH 8.8) with urea (6 M), glycerol (30 %, v/v) and SDS (2 %, w/v) buffer containing dithiothreitol at 10 mg/ml or iodoacetamide at 25 mg/ml. SDS-PAGE was performed using 12.5% (w/v) duracrylamide gels, followed by staining with Coomassie Brilliant Blue GR 250 (Consoli and Damerval 2001).

MALDI-TOF analysis

Proteins were identified by MALDI-TOF/TOF (model 4700 from Proteomics Analyzer ABI) and/or by liquid-mass chromatography (CapLC-Q-TOF; Waters/Micromass). Protein samples isolated from Coomassie-stained 1D or 2D gels were destained, reduced, alkylated, and finally digested with sequencing-grade modified trypsin (Pandey et al. 2000). Criteria used to accept the identification were significant homology scores achieved in Mascot, a minimum of four peptides matched and a protein sequence coverage greater than 10%.

Transglutaminase activity

TG activity was determined by measuring the incorporation of [1,4(n)-^{3}H]-putrescine (Put; specific activity 962 GBq/mmol) into *N*,*N*-dimethylcasein (Bernet et al. 1999). Samples were repeatedly precipitated and the radioactivity was measured in a scintillation counter (Beckman LS 6000 SC, Fullerton, CA). For the assays with illumination, we used a white-light intensity of 90–100 μmol m^{-2} s^{-1}. The final concentrations of TG inhibitors used were as follows: GTP, 1 mM; monodansyl cadaverine (MDC), 1 mM; Boc-DON-Gln-Ile-Val-OMe (B003; N-zyme), 1 μM.

Refolding of TGZ4p

Sixteen experiments were performed using the Foldlt Screen kit (Hampton Research; Armstrong et al. 1999), which evaluates 12 factors in 16 unique solutions included in the Foldlt kit. Briefly, aliquots of TGZ4p, at 0.1 mg/ml or 1 mg/ml, were incubated in the different Foldlt solutions for 6 h at 4°C with agitation at 4 rpm. Solutions were dialyzed against 100 mM Tris/HCl, pH 8.0 at 4°C to remove the Foldlt reagents and precipitate unfolded proteins. The supernatants were then assayed for correctly folded protein using the TG-activity assay described above. A 30 ml refolding assay was performed using Foldlt condition 9 [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] and 0.1 mg TGZ4p/ml.

Results and discussion

TGZ variants differing in number of B-repeats generated via PCR subcloning

As an antecedent of the present results, we previously cloned two cDNAs coding for maize chloroplast transglutaminases (Villalobos et al. 2004), which differ in the number of B-type repeats within the central, non-catalytic domain, *TGZ15* (15 repeats) and *TGZ21* (21 repeats), see Fig. 1.

The sequence of the DNA product obtained from PCR cloning was identical to the original nucleotide sequence, but with only four tandem B-repeats. This

truncated variant was named *TGZ4*. The loss or gain of nucleotide repeats without affecting surrounding sequences, as observed here with the truncated version TGZ4, could be explained by an event termed mega-priming. Repetitive elements that are either incompletely synthesized or broken during PCR can anneal and then extend, resulting in repeat length variation independent of polymerase type or PCR conditions (Clarke et al. 2001). Using different primers and similar PCR conditions, two other constructs were generated, *TGZ9* and *TGZ29*, both lacking the *N*-terminal sequence corresponding to the putative chloroplast transit peptide. The original *TGZ15* sequence was also subcloned into the pET28-b (+) vector using appropriate restriction enzymes. The deduced amino acid sequence of these four constructs is presented in Supplemental Fig. 1.

Neither the chloroplast transit peptide nor repeat number affect TGZ expression

To analyze the possible relevance of repeat number and of the chloroplast transit peptide on TGZ overexpression and activity, all four constructs described above were used for overexpression experiments. After IPTG induction and treatment with CelLytic reagent, soluble and insoluble protein fractions were analyzed by SDS-PAGE and Western blot using the anti-T7·tag antibody (Fig. 2A).

In all cases, the majority (>80%) of recombinantly expressed TGZ was found in the inclusion body (IB) fraction. Although the TG activity of all the different constructs was not measured, none of the repeats found in *TGZ21* or *TGZ15* possesses a cysteine residue that could act as nucleophile. Furthermore, in a BLAST search against the Gramene Database (http://www.gramene.org/Oryza_sativa/index.html), we identified a rice DNA sequence (AL606595) highly similar to *TGZ15* (87% identity at DNA level), and with only three, incomplete, B-repeats. Altogether, it would seem that the central TGZ domain is not involved in catalysis. This is also supported by studies on coagulation factor V from different species, which presents a significant homology to the repeat domain of *TGZ15* and *TGZ21*. The removal of B-type repeat sequences (varying in number from 35 in human to 41 in the pig cofactor) does not compromise production of active factor V as a recombinant protein (Kim et al. 2000).

All recombinant polypeptides (*TGZ4p, TGZ9p, TGZ15p* and *TGZ29p*) of the expected M_r were also identified as *Zea mays* L. TG by MALDI-TOF analysis of trypsin digests. The anti-T7·tag antibody recognized additional minor bands of lower M_r in the over expressed TGZ4p inclusion bodies, which were also identified by MALDI-TOF as TGZ, indicating proteolytic breakdown (Fig 2B). From these results, it appears that the proportion of soluble recombinant protein was not influenced by the presence or absence of the chloroplast transit peptide, or by the number of B-type repeats in the expression construct. Comparison of soluble (S) and IB fractions by Western blot (Fig. 2A), revealed ~12% (w/w) soluble TGZ4p fraction, and when cells were grown in with 0.5 M NaCl (data not presented), this fraction increased up to 25%. Consequently, further experiments were performed with the IB fraction of the minimal cloned plant transglutaminase, TGZ4p.

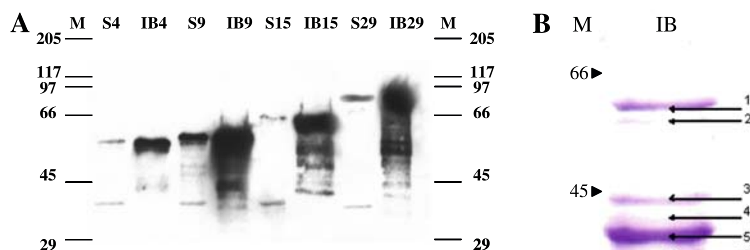

Fig. 2 Overexpression experiments using different TGZ constructs. (**A**) Western blot analysis of the soluble (S) and IB fractions; numbers 4, 9, 15 and 29 correspond to the number of B-repeats in the protein (TGZ4p, TGZ9p, TGZ15p, TGZ29p). (**B**) SDS-PAGE analysis of proteins stored in inclusion bodies (TGZ4p). Bands 1, 2, 3, 5 and 6 were identified by MALDI-TOF analysis as TGZ, while band 4 corresponds to *E. coli* porin OmpF

TGZ4p purification

TGZ4p isolated from the cytosolic cell fraction under native conditions co-purified with other host proteins (Fig. 3A, NE) and gave low yields of soluble recombinant TGZ4p. Given the limited success of native purification, subsequent efforts were directed to the purification and renaturation of TGZ4p from urea-solubilized IBs. To this aim, IBs solubilized in 8 M urea and purified on small-scale Ni-NTA columns gave notably higher amounts of His-tagged TGZ4p, and of much higher purity, than the soluble fraction (Fig. 3A, DE). When aliquots of affinity-purified TGZ4p (native and denatured) were dialyzed and centrifuged, although part of the recombinant protein purified under denaturing conditions precipitated, the soluble fraction was found to have transglutaminase activity (Fig. 3B), indicating that a significant amount of protein had been correctly refolded. This finding strongly suggests that TGZ4p purification from inclusion bodies under denaturing conditions, followed by a refolding step, is a feasible way to produce active TGZ4p for further biochemical characterization.

In order to obtain higher quantities of purified protein, recombinant protein from a 1 l culture was purified by FPLC under denaturing conditions, as described in Materials and methods. The final chromatographic peak contained a major protein band with a M_r of $\sim$55 kDa (Fig. 3C, fr 73), identified as maize transglutaminase. Purified fraction 73 was also analyzed by 2D gel electrophoresis to evaluate the degree of TGZ4p purification achieved (Fig. 3D). In both cases, the obtained proteins were analyzed by MALDI-TOF, and it was concluded that the degree of purification achieved was adequate for the subsequent refolding experiments.

Purified TGZ4p was also used to generate a polyclonal antibody, which efficiently recognizes TGZ4p in transformed *E. coli* cells as efficiently as TGZ in maize chloroplast extracts (Fig. 4, A and B).

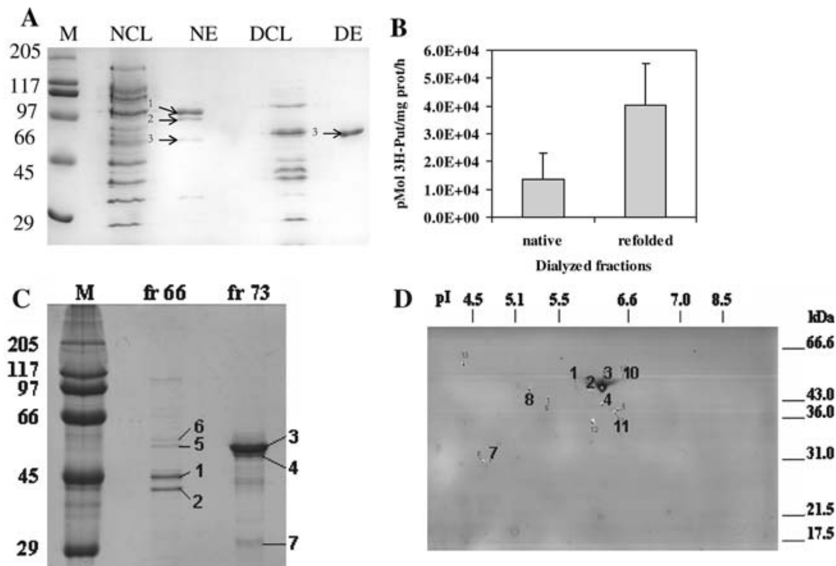

Fig. 3 Purification of recombinant TGZ4p. (**A**) Small-scale purification of both native and urea-denatured TGZ4p. NCL, native cell lysate; NE, native eluate; DCL, denatured cell lysate; DE, denatured eluate. Numbers refer to proteins identified by MALDI-TOF analysis: 1, putative formyltransferase (*E. coli*), GI: 16130190, 74.87 kDa; 2, unnamed protein product (*E. coli*), GI:43268, 67.05 kDa; 3, transglutaminase (*Zea mays*) GI: 47678879, 54.49 kDa. (**B**) TG activity of the small-scale purified TGZ4p fractions dialyzed against 20 mM Tris/HCl, 0.5 M NaCl, pH 8.0, assessed by measuring the incorporation of radiolabeled putrescine to substrate DMC. Data are the means ± SD of experiments performed in triplicate. (**C**) 1D SDS-PAGE analysis of FPLC peaks named as fractions 66 and 73. Bands 3 and 4 were identified as maize TG and other numbered bands as host contaminants. M, Molecular weight marker (kDa). (**D**) 2D analysis of fraction 73. Spots 1, 2, 3, 4, 10 and 11 were identified as maize TG, while spots 7 and 8 correspond to host contaminants

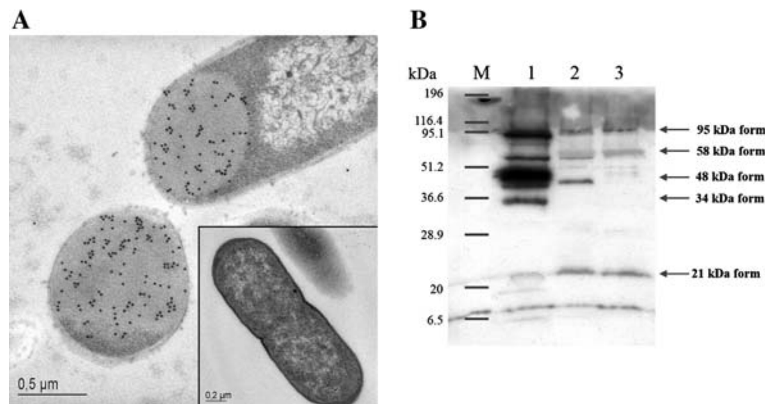

Fig. 4 (**A**) subcellular immunolocalization of TGZ4p by TEM with anti-TGZ4p polyclonal antibody obtained from purified TGZ4p (1:3000). Samples were incubated with the secondary antibody conjugated with 15 nm gold particles. Observations were performed with a TEM Jeol JEM 1010 at 80 kV. The inset in the panel shows non-transformed *E. coli* cell as a control. (**B**) TGZ immunolocalization by western blot using the anti-TGZ4p in maize thylakoid extracts from plants growing in different light conditions, lane 1 = etiolated plant, lanes 2 and 3 = plants growing under light conditions for 3 and 20 days respectively in the greenhouse. M = Molecular marker. Arrows show different forms of chloroplastic maize TG

Recombinant TGZ4p can be effectively renatured in vitro and exhibits general TG enzyme characteristics

The fact that TG activity could be recovered by dialysis of the urea-denatured IB fraction (see above) prompted us to perform a systematic search of conditions that support enzyme refolding. Using the soluble fraction after dialysis, the highest levels of incorporation of ^{3}H-labeled Put to substrate casein, indicating correct folding of TGZ4p, were found using conditions 5, 9 and 12 (Fig. 5A).

A statistical analysis (multiple range test) of these results indicated that the presence of sucrose and high ionic strength were the most positive folding variables (Fig. 5B). Basic pH (8.2) was negative for the folding process and also the presence of DTT and detergent had a slightly negative effect. Protein concentration did not appear to have any effect on TGZ4p folding. Further refolding experiments with Foldlt conditions 5, 9 and 12 and a fixed protein concentration (0.1 mg/ml), confirmed that condition 9 yields the highest activity, with 60% (w/w) of the starting TGZ4p recovered after dialysis (Fig. 5*C*). The composition of this solution fits optimally with the model suggested by the main effect factor analysis (Fig. 5B), as it contains sucrose (440 mM), high NaCl/KCl concentrations (264 and 11 mM, respectively), and has an acidic pH (6.5).

The TGZ4p incorporated ~190 pmol Put (mg DMC)$^{-1}$ h^{-1} (Fig. 5C), giving an activity 1600-fold higher (using the same polyamine as substrate) than the figure reported for recombinant AtPgn1p (*Arabidopsis* sp.), the only plant enzyme with TG activity expressed to date in *E. coli* (Della Mea et al. 2004). In this case, however, the recombinant protein yield was not reported. The differences observed between TGZ4p and AtPng1p might be due to intrinsic enzyme characteristics, such as substrate preferences as well as different subcellular localization as AtPgn1p was detected in the microsomal-enriched fraction while TGZ4p is localized in chloroplasts.

To further characterize refolded TGZ4p and verify that it was a functional transglutaminase, we assayed the inhibitory effect of different compounds on its transglutaminases activity (Fig. 5D). When the enzyme reaction was performed in the absence of Ca^{2+}, or with GTP (1 mM), MDC (1 mM) or the tissue TG inhibitor, Boc 1 μM (Hausch et al. 2005), ^{3}H-Put incorporation to substrate DMC mediated by TGZ4p was significantly lower than that of the positive control (DMC incubated alone), and similar to blank controls. It was also found that illumination during the enzyme assay did not increase chloroplastic TGZ4p activity when DMC was used as substrate. Similar results were obtained with TGZ15p and TGZ21p (Villalobos et al. 2004).

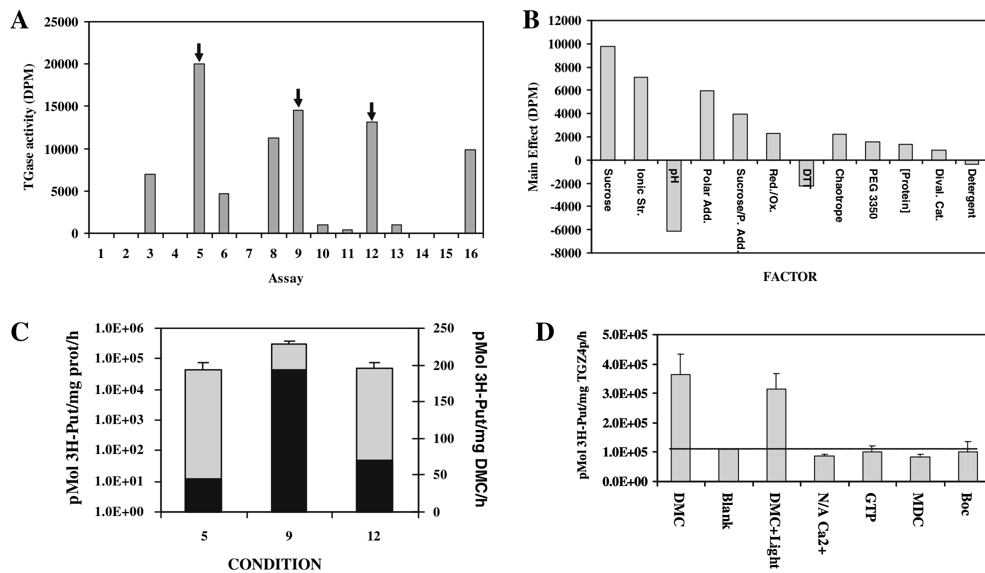

Fig. 5 Refolding of TGZ4p. (**A**) Transglutaminase activity of soluble material (given in DPM) after the screening for TGZ4p refolding conditions using the FoldIt Screen kit (Hampton Research). Black arrows indicate the assays selected. (**B**) analysis of major factors influencing TGZ4p refolding. The main effects for each factor were calculated as (Σ''+ level''–Σ ''– level'')/*n*, where *n* is the number of experiments (Statgraphics Plus Version 4 software). Add = additive, P = polar. (**C**) TG activity assay (expressed as ^{3}H-Put incorporation per mg of refolded protein or per mg of DMC) of TGZ4p samples obtained using the three refolding conditions selected in **A**. (**D**) Levels of ^{3}H-Put incorporation to DMC by refolded TGZ4p, under different conditions. DMC, control sample; Blank, boiled and TCA-precipitated sample; DMC + light, illumination during the assay; N/A Ca^{2+}, no calcium ions added to the reaction mix; GTP, 1 mM guanosine triphosphate added; MDC, 1 mM mono dansyl cadaverine added; Boc, 1 μM Boc-DON-Gln-Ile-Val-OMe (N-zyme) added

Conclusion

We have shown that a maize-derived TG can be efficiently produced in *E. coli*. Although the majority of TGZ4p was found in the insoluble, inclusion body fraction, we were able to obtain high yields of active enzyme with the biochemical characteristics described for other TGs by refolding of urea-extracted IBs. Pure and concentrated recombinant TGZ4p is a valuable tool for further studies on structure and function. Research is in progress on the industrial applications of TGZ, based on its expression in 5-l bioreactor scale and in other heterologous systems.

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