The Activity of the Durum Wheat (*Triticum Durum* L.) Catalase 1 (TdCAT1) Is Modulated by Calmodulin

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Abstract

Background: In plant cells, Catalases (CAT) are involved in the scavenging of the reactive oxygen species during development processes and in response to abiotic and biotic stresses. However, little is known about the regulation of the CAT activity by cations and calmodulins (CaMs).

Methods and Results: Using bio-informatic analysis and in vitro catalase assays, we showed that durum wheat catalase 1 (TdCAT1) harbors highly conserved cation-binding domains which are localized at different positions of the protein. Moreover, the protein exhibits a CAT activity in vitro that is specifically enhanced by Mn^{2+} and Fe^{2+} and to a lesser extent by Cu^{2+}, Zn^{2+} and Mg^{2+} but not with Cd^{2+} cations. Moreover, we showed that TdCAT1 harbors a conserved calmodulin binding domain (CaMBD) and binds to CaMs in a Ca^{2+}-independent manner via a GST-pull down assay. Moreover, TdCaM1.3/Ca^{2+} complex stimulated the catalytic activity of TdCAT1 in a CaM-dose dependent manner. Most interestingly, addition of Mn^{2+} cations enhance TdCAT1 activity in presence of CaM/Ca^{2+} complex.

Conclusion: The catalase activity of TdCAT1 is enhanced in presence of various divalent cations. Besides, TdCaM1.3 proteins stimulates the catalase activity of TdCAT1 in Calcium dependent manner. Such effects were not reported so far and raise a possible role of CaM and cations in the regulation of plant CATs during cellular response to external signals.

1. Introduction

Reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (^-OH), are toxic products of the normal oxygen (O_2) metabolism. In fact, they cause serious damages to vital macromolecules (proteins, lipids, and nucleic acid) by inducing oxidative stress [1]. Moreover, ROS acts as second messenger and it is produced after plant exposure to different stresses such as water deficit, salinity, and extreme temperatures [1]. Some studies revealed that those signaling molecules are crucial for maintaining normal cellular functions [2] and they mediate cell death rather than to kill cells previously demonstrated [3]. In plant cells, ROS are primordial for the function of biological processes at low intracellular levels, whereas at elevate levels they become toxic [4]. Thus, cells must maintain a constant basal level of ROS to ensure ROS signaling by controlling the balance between production and removal of ROS [5]. ROS scavenging pathway in plants is ensured by different enzymes such as superoxide dismutases (SODs), peroxidases (PODs) and catalases (CATs) [6], which regulate a SOD reaction [7].

Catalase (CAT), a tetrameric heme-containing enzyme, plays a crucial role in plant growth, maturation, fruit ripening, postharvest events, and stress responses [8–9]. It is one of the most important components of the cell defense mechanism that removes oxidative stress (ROS; 9) and plays a crucial role in the antioxidant response system by removing the excessive H_2O_2 generated during developmental processes or by environmental stimuli into water and oxygen in all aerobic organisms [9]. In higher plants, catalase was reported to be localized in peroxisomes of leaves, glyoxysomes, roots, cotyledons as well as

unspecialized peroxisomes [7, 9]. Some studies showed that CAT is localized in the mitochondria [10]. CATs present four identical subunits [8]. Unlike animals, which harbor only one gene encoding for CAT, plants genomes encode for multiple isozymes and their numbers varies depending on the species [9]. For example, tobacco (*Nicotiana plumbaginifolia Viviani*), maize (*Zea mays*), Arabidopsis thaliana, and rice (*Oryza sativa*) genomes harbor 3 different genes each encoding for catalase isozyme [11]. However, there are two catalase genes in barley (*Hordeum vulgare*), and peach (*Prunus persica*)[12]. Several proteins were reported to interacts with catalase such as salt overly sensitive protein (SOS_2) [13], nucleoside diphosphate kinase 1 (NDK1) [14] triple gene block protein 1 (TGBp1) [15] and LESION SIMULATING DISEASE1 (LSD1) [16]. In Arabidopsis, it has been demonstrated that small heat shock protein Hsp14.6CIIinteracts with catalase AtCAT2 in the cytosol of *Arabidopsis* cells [17], as well as in the peroxisomes, and this interaction increases the catalytic activity of the catalase in NAC1 dependent manner in vivo [18]. Moreover, Hsp14.6CII/AtCAT2interaction prevents the catalase from thermal aggregation [18].

Calmodulin (CaM) is one of calcium sensors [19] that perceive transient changes in cytosolic Ca^{2+} levels and rapidly participates in response to diverse stimuli in plants [20] by regulating different cellular processes, such as biotic and abiotic stress responses and plant growth and maturation [21]. CaMs are small acidic proteins (148 aa) that are highly conserved in eukaryotic cells in both nucleic and protein sequences [22]. They bind four Ca^{2+} ions with high affinity for calcium binding domains called EF-hand motifs [23] arranged in N- and C-terminal globular domains [24]. Upon binding to Ca^{2+}, CaM changes conformation from a closed, Ca^{2+}-freestate (apoCaM) to an extended Ca^{2+}/CaM conformation. This conformational change allows to hydrophobic surfaces surrounded with negative charges to be exposed to target proteins with high affinity [19, 24]. This structural flexibility, along with the ability of some proteins to interact with CaM independently of Ca^{2+}, allows CaM to regulate numerous protein targets implicated in a huge range of cell responses such as cold, wind, wounding, pathogenic attacks, and gene regulation [25]. Many works reported the interaction of CaMs with an important number of proteins. In fact, it is estimated that around 300 proteins are able to bind to CaMs in plants [25] such as from durum wheat Pathogen Related protein (PR-1) [26]; MAP Kinase Phosphatase [27], transcription actors [28], as well as CAT proteins as revealed in potato [29], *Arabidopsis* [30], and sweet potato [31].

In a previous work, TdCAT1 from durum wheat was identified and functionally characterized [32]. TdCAT1 harbors a putative calmodulin binding domain (CaMBD) that is localized at its C-terminal part (413-453 aa) [32]. This domain was reported to be essential for calmodulin binding and activation of some plant CATs in calcium dependent manner [30]. In this study, we provided experimental evidence that durum wheat TdCAT1 activity is enhanced by the presence of Mn^{2+} and Fe^{2+}. Moreover, TdCAT1 binds to TdCaM1.3via a conserved calmodulin binding domain localized at the C-terminal part of the protein. Besides, the TdCaM1.3/Ca^{2+} complex stimulate the catalase activity of TdCAT1 either alone or in the presence of Mn^{2+} and Fe^{2+}. These data were never described so far and suggest a possible contribution of calmodulin and Mn^{2+} in the modulation of durum wheat CAT activity in plant cells.

2. Material And Methods

2.1. Bioinformatic analyses

Calmodulin binding domains were revealed by the calmodulin target database(http://http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html) [33]. Cation binding domains were investigated using uniprot database[34], Supfam databases (http://supfam.org/SUPERFAMILY/cgi-bin/align.cgi) [35], and swiss model database, (https://swissmodel.expasy.org/interactive/T5XR77/models/) [36] for the identification of Mn^{2+}/Mg^{2+}, Ca^{2+}, Zn^{2+}/Cu^{2+} and Fe^{2+} binding domains respectively. HMMER database [37]was used for analyzing the functional domains present in catalase sequence.

2.2. Production and purification of recombinant TdCAT1 proteins and its truncated forms in E. coli

To produce the recombinant proteins His_TdCAT1 and the different truncated forms [His_TdCAT_{200} (1-200aa); His_TdCAT_{295} (1-295aa); and His_TdCAT_{460} (1-460aa)], each product was amplified by PCR with the Pfu Taq DNA polymerase and using the appropriate primers (Table 1), digested by the appropriate restriction enzymes, *EcoR*I and *Xho*I, and cloned in-frame with an Histidine- tag into the pET28a expression vectors (Novagen, Madison,WI, USA) into *EcoR*I and *Xho*I restriction sites. The same procedure was also conducted to produce the recombinantHis_TdCaM1.3 (Accession N° MW057248). The product was amplified by PCR with the Pfu Taq DNA polymerase in the presence of the appropriate primers (Table 1), containing *EcoR*I restriction sites then digested and cloned in-frame with a Histidine-tag into the pET28a expression vectors.

The resulting constructs pHis_TdCAT1, $pHis\_TdCAT_{200}$, $pHis\_TdCAT_{295}$, $pHis\_TdCAT_{460}$, and His_TdCaM1.3 were introduced into the BL21 *E. coli* strain (DE3) (Novagen). A single selected colony from each construction was grown overnight at 37°C in LB medium containing 100 µg/ml Kanamycine with shaking at 220 rpm. The culture was next diluted 1:100 into fresh LB-Kanamycine medium and grown to an OD of 0.6 at 600 nm. Protein expression was then induced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for overnight at 37°C. Bacterial cells were harvested by centrifugation at 4500 rpm for 10 min at 4°C and the pellets were subsequently washed twice with cold water. Later, the cells were harvested in cold lysis buffer (Tris-HCl 100 mM pH 8; EDTA 1 mM; NaCl 120 mM; 1mM DTT, 50 mM PMSF and 0.5% Tween) and sonicated on ice. Afterwards, the cells were centrifuged at 9000 rpm for 45 min at 4°C. The deleted forms were purified from the supernatant whereas pHis_TdCAT1 was not found in the supernatant. Thus, the recovered inclusion bodies were resuspended and incubated in the lysis buffer for overnight at 4°C with agitation then centrifuged at 9000 rpm at 4°C for 10 min. The supernatant was then loaded on Ni-Sepharose column (Bio-Rad) pre-equilibrate with binding buffer (Tris-HCl 100 mM pH 8; NaCl 0.5 M; 30 mM imidazole) and gravity eluted.

In another hand, the durum wheat calmodulin proteinTdCaM1.3 cloned in frame with GST was expressed and purified as previously described [26]. Protein quantification was performed using the Bradford method [38] and the correct size of recombinant proteins was checked by SDS-PAGE electrophoresis.

2.3. CAT activity assays

CAT activity was determined according to Feki et al. [39]. CAT activity was measured spectrophotometrically at 240 nm using a specific absorption coefficient at 0.0392 cm^2 μmol^{-1} H_2O_2 1 mL of substrate solution made up of 50 mM H_2O_2 in a 75 mM phosphate buffer at pH 7.0 and 160 µg of proteins were mixed at 25°C for 1 min and reaction was stopped by adding 0.2 mL of 1 M HCl. CAT activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease in absorbance at 240 nm and was calculated as µmol H_2O_2 decomposed/mg protein/min. Catalase activities were also assayed by measuring the amount of H_2O_2 present in the reaction medium in the presence or absence of calmodulin as well as bivalent cations such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+} and Cu^{2+}.

2.4. Biochemical characterization of the catalase TdCAT1

To investigate the effect of pH on the catalase activity of TdCAT1, the purified His-TdCAT1 was incubated at room temperature for 10 min in various buffers prior to catalase assays. Catalase assays was measured within a range of pH from 3.0-9.0 using 75 mM buffers. For the effects of temperature, the standard reaction mixtures were pre-incubated at the optimum pH at the indicated temperature range (10-80°C) for 10 min and then they were assayed for catalase activity.

2.5. GST-Pull down assays

Prior to binding, Glutathione Sepharose 4B beads were washed with the appropriate Tris-HCl buffer (Tris-HCl 20 mM; pH 7.4, EDTA 1 mM, DTT 0.5 mM, NaCl 150 mM, 0.5% Triton, PMSF 1 mM) then the same buffer was used to equilibrate those beads. After that, the beads were incubated with 12 µg of GST_TdCaM1.3 or GST alone for 2h at 4°C and washed three times to discard the unfixed proteins. Twenty micrograms of the different recombinant forms of His_TdCAT1 proteins were then incubated with the immobilized proteins overnight at 4°C. After extensive washes, proteins were dissociated from the beads by boiling in Tris-HCl 50 mM, pH 6.8, DTT 1 mM, SDS 2%, glycerol 10%, bromophenol blue 0.1% then separated by SDS-PAGE (10%). The His-TdCAT1 and the other deleted forms were finally detected by western blot using the anti-Histidine antibody (Sigma) as described by the manufacturer.

2.6. Statistical analysis

Differences between enzymatic reactions in presence of catalase alone or with Calmodulin and/or cations were analyzed by two-way ANOVA comparison tests with statistical significance set at $\alpha < 0.05$ relative to the control [40].

3. Results

3.1. Determination of TdCAT1 activity

After recovery from the inclusion bodies, the recombinant His_TdCAT1 was purified using Ni-sepharose column chromatography (Fig. 1a, b). It is known that the activity of CATs towards their substrate is very low [41]. Thus, the optimum buffer concentration was also studied in presence of different buffer concentrations (25, 50, 75 and 100 mM phosphate buffer). These results showed that saturating buffer concentration is 75 mM. Moreover, the effect of pH upon TdCAT1 activity in the decomposition of H_2O_2 was studied. The pH selected ranged from 3.0 to 9.0. Below pH 5 the activity of TdCAT1 was very low (Fig. 1c). The reaction of CAT toward H_2O_2exhibits remarkable pH stability; negligible change is measured from pH 3 to 5. Moreover, this activity showed a sharp optimum at pH =7 in presence of 160 µg of recombinant His_TdCAT1 as a minimum quantity with which we register a significant activity. Thus, for the rest of the experiments, the pH was fixed at 7.

For the optimum temperature, we performed a series of CAT activity assays using different temperatures 10 to 80°C. Our results show that the catalase activity is important at 25°C and decreases gradually with temperature (Fig. 1d). This activity depends on the tertiary structure of the protein since protein denaturation by heat treatment completely abolishes the CAT activity (data not shown). Thus, in this study the optimum buffer concentration, pH, and temperature for TdCAT1 were determined as 75 mM, 7 and 25°C, respectively.

In a second step, we determined the initial reaction rate (Vo) by measuring enzyme's kinetics of the purified recombinant proteins His_TdCAT1 during the first min. As it is known that CAT activities can be modulated by bivalent cations [42], and the registered activity in our experimental assays was relatively low (96.27 µmol/min/mg of protein), we investigated whether His_TdCAT1 needs divalent cations to enhance its activity. For this purpose, different enzyme assays were performed with TdCAT1 in the presence of 2 mM of Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+} or Cu^{2+}. Experimental results showed that the catalytic activity is significantly stimulated in the presence of 2 mM Mn^{2+} and Fe^{2+} and with a lesser extent by Zn^{2+}, Cu^{2+} and Ca^{2+} and slightly by Mg^{2+} (Fig. 1e). In contrast, this activity was not significantly modified by Cd^{2+} (Fig. 1e). Thus, a dose response assay was performed with these cations separately and the results showed that the activity of TdCAT1 is enhanced by increasing Mn^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} or Mg^{2+} concentrations. In fact, the maximal activity of TdCAT1 (about 16-fold higher than in control conditions) was reached using 1 mM Fe^{2+} (Fig. 2a) or Mn^{2+} (Fig. 2b). In the presence of those cations, the catalytic activity of TdCAT1 started to increase with 0.5 mM of both cations (about 3-fold; Fig. 2a, b). Interestingly, the same result was observed in presence of Ca^{2+} cations. In fact, the catalase activity of TdCAT1 was stimulated 5 times than in absence of calcium (Fig. 2c). This stimulation started with 1 mM Ca^{2+} and reached its maximum in presence of 2 mM Ca^{2+}. In another hand, in presence of Zn^{2+} and Cu^{2+}, the catalytic activity of TdCAT1 increases with 0.5 mM of both cations (about 8-fold increase) and reached to the maximum in presence of 3 mM (Suppl. Fig. 1a, b). In the presence of Mg^{2+}, the activity of TdCAT1 also increases in a dose-dependent manner, but to a lesser extent than with Mn^{2+}, Fe^{2+}, Zn^{2+},

Cu^{2+}, and Ca^{2+} (Suppl. Fig. 1c). The stimulation started to increase in presence of 0.5 mM Mg^{2+} and reached its maximum of 3.2-fold increase using 3 mM Mg^{2+} compared to the basal activity (Suppl. Fig. 1c). The effects of all those cations were exerted gradually with no fold induction. Therefore, Mn^{2+} and Fe^{2+} appear more efficient than other cations (Mg^{2+}, Zn^{2+}, Cu^{2+}, and Ca^{2+}) on the TdCAT1 activity *in vitro*. Thus, those cations were used to perform the rest of experiments. Altogether, these results showed that the catalase activity of TdCAT1 increase in a gradual manner by increasing cations concentration in the medium. It is worth to note that cation stimulation is specific and not artifactual since no activity could be detected when similar assays are performed in absence of enzyme or when we used a heat denatured form of His_TdCAT1 incubated at 100°C for 10 minutes (data not shown).

3.2. TdCAT1 harbors conserved ion binding motifs required for its activations by divalent cations at different parts of the protein

The observed stimulatory effects of Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} on TdCAT1 activity suggest that durum wheat CAT may harbor cation binding motifs. Thus, we analyzed the structure of TdCAT1 to identify potential cations binding domains. Alignment with well-known Mn^{2+} or Mg^{2+} binding proteins (http://www.uniprot.org) revealed the presence of putative Mn^{2+}(position 44-55) and Mg^{2+} (position 439-449) binding sites on the N-terminal and the C-terminal region of TdCAT1 sequence respectively (Fig. 3a). These domains are highly conserved among CATs proteins from different plant species (Suppl. Fig. 2a, b). Moreover, we identified putative Copper/Zinc binding domain, Calcium binding domain and Iron binding domain in the sequence of TdCAT1. In fact, the analysis of TdCAT1 amino acids sequence revealed that this protein contains H-$(X)_{12}$ –H type motif, known as a cupper binding domain, which is localized at 165-177aa position in the TdCAT1 sequence (HIQENWRILDLFSH, Fig. 3a). In another hand, sequence investigation of zinc binding domains shows that TdCAT1 contains a domain homolog to the domain identified in protein AN1. These motifs bind a single zinc atom (the European Bioinformatic Institute: https://www.ebi.ac.uk/interpro/potm/2007_3/) and is localized at the amino acids 163-191 position. This motif is related to AtSAP10 protein (Q9STJ9). Thus, TdCAT1 harbors a Zn/Cu binding domain located at its N-terminal region (163-191aa; Suppl. Fig. 3). Moreover, using the Swiss model database, (https://swissmodel.expasy.org/interactive/T5XR77/models/), we found a degenerate domain for iron binding (HDV domain) located at 76-85aa position with conservation of Histidine residue implicated in iron binding. This domain is also well conserved in all studied CATs (data not shown). Finally, using the Supfam databases (http://supfam.org/SUPERFAMILY/cgi-bin/align.cgi), a Calcium binding domain called EF-hand was identified in the sequence of TdCAT1. The consensus pattern for calcium-binding is D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]. In TdCAT1, this domain is localized at amino acids position 266-293 (Suppl. Fig. 4).

To confirm the presence of those putative cation binding domains, we generated three different deleted forms which are $TdCAT_{200}$ (containing the first 200 aa), $TdCAT_{295}$ (containing the first 295 aa), and

$TdCAT_{460}$ (containing the first 460 aa) (Fig. 3b). After production and purification of those forms, we measured their catalytic activities in absence of divalent cations. Interestingly, $TdCAT_{200}$ has a very week basal activity (4.011 µmol/min/mg of protein) whereas $TdCAT_{295}$ has a better catalytic activity (29.41 µmol/min/mg of protein) while $TdCAT_{460}$ and the non-truncated protein TdCAT1 have the same catalytic activity (96.27 µmol/min/mg of protein) (Fig. 3c). This result could be explained by the fact that TdCAT1 protein contains one catalase domain (Pfam Id PF00199, 18-399 aa) and catalase related immune-responsive (Pfam Id PF06628.11, 421- 486; data not shown) as revealed by HMMER database. Those results were also shown for some OsCATs demonstrating that the presence of whole catalase domain is essential for protein activity [41]. As a result, the entire catalytic domain is important for the catalytic activity of TdCAT1.

To confirm the presence of these cation binding domains, the different deleted forms were used to perform CAT activity tests. We measured the catalytic activities of those forms in presence of divalent cations. We first used the $TdCAT_{200}$ form that contains the Mn^{2+}, Cu^{2+}/Zn^{2+} and Fe^{2+} binding domains (Fig. 3b). As expected, $TdCAT_{200}$ activity was stimulated by Mn^{2+}, Fe^{2+}, Cu^{2+} andZn^{2+} but not with Ca^{2+} and Mg^{2+} (Fig. 4a) confirming that the Ca^{2+} and Mg^{2+} binding domains are not located in the first 200 aa while Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} binding domains could be present in those first 200 aa. Similarly, $TdCAT_{295}$ was stimulated by Mn^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+} and Zn^{2+} but not with Mg^{2+} (Fig. 4b) while $TdCAT_{460}$ was stimulated also by Mg^{2+} (Fig. 4c). Those results suggested strongly that TdCAT1 contains 5 different putative cations binding domains that are localized at different parts of the protein (Fig. 3a).

3.3. TdCAT1 harbors a conserved calmodulin binding domain

Similarly, to other plant catalases, TdCAT1 protein contains a putative CaMBD located at the C-terminal region of TdCAT1 positioned from residues 413 to 453 [32]. Protein sequence analyzes by the Calmodulin target database (http://calcium.uhnres.utoronto.ca)server revealed that this domain is rather located at 459-482 aa (Fig. 5a). Moreover, in silico analyzes using interpret server (https://www.ebi.ac.uk/interpro/entry/IPR000048) reveals a putative interaction between TdCAT1 and CaMs. The construction of the helical wheel model showed that this CaMBD have basic and hydrophobic amino acids (53.54%, Fig. 5b) segregated into the opposite side of the helix, which has been named the basic amphiphilic_α helix (Baa) motif (data not shown).

3.5. TdCAT1 interacts in vitro with TdCaM1.3

Like other plant catalases, TdCAT1 protein has a putative CaM binding motif located at the C-terminal part of the protein. To characterize the regulation of TdCAT1 by CaMs, we investigated the CaM binding properties of TdCAT1. To this purpose, in vitro GST-pull down assay was performed using TdCaM1.3 protein as bait as previously described [26]. To perform a pull-down assay, the recombinant proteins His_TdCAT1, His_TdCAT_{200} and GST-TdCaM1.3were first purified (Fig. 6a, b). Then, the purified His_TdCAT1 was mixed with the Nickel beads bound GST_TdCaM1.3 and the interaction between the two

proteins were investigated by immune blotting the membrane using the anti-Histidine antibody. As shown in Fig. 6c, the His_TdCAT1 was pulled down by the GST_TdCaM1.3 (lane 3) but not with beads alone (lane 1) while the GST_TdCaM1.3 was not detected using the anti-histidine tag antibodies (lane 2). This interaction appears to be Ca^{2+} independent since the signal corresponding to TdCAT1 is detected when the GST_TdCaM1.3 was supplemented with 2 mM Ca^{2+} (Fig. 6b, lane 4) and or in the presence of the chelating agent EGTA (5 mM) (Fig. 6b, lane 5). Thus, TdCAT1 harbors a conserved calmodulin binding domain and interacts with CaMs in calcium independent manner contrary to other identified catalases isolated from Arabidopsis and potato. To confirm this result, the truncated form His-$TdCAT_{200}$(contains no putative CaMBD) was used for pull down assays. As expected, His_$TdCAT_{200}$which has lost the C-terminal part including putative CaM binding domain, couldn't be pulled down by GST_TdCaM1.3 (Fig. 6d) confirming that TdCAT1 interaction to CaM is specific and requires the conserved CaM-binding motifs.

3.6. Effects of TdCaM1.3 on TdCAT1 activity

It has been demonstrated that CaM proteins interact with various target proteins and modulate their activities [22, 26, 27, 43]. Consequently, we investigated the effect of wheat calmodulin binding on the catalase activity of TdCAT1 using in vitro assays. As shown in Fig. 7a, in the absence of Ca^{2+}, TdCaM1.3 alone did not modify TdCAT1 activity. Thus TdCaM1.3 alone had no effect on catalase activity of TdCAT1. In the presence of calcium, both His-TdCaM1.3 (Fig. 7b) and GST-TdCaM1.3 (data not shown) stimulated the catalytic activity of TdCAT1 with the same fold and it reached the maximum in the presence of 2 mM of Ca^{2+}. Thus, for the rest of the catalase assays, we used His-TdCaM1.3. TdCAT1 activity started to increase with Ca^{2+} concentrations as low as 0.5 mM and stimulation reaches its maximum level using 2 mM of Ca^{2+} corresponding to 8 times increase of the Vo in presence of TdCaM1.3 (Fig. 7b). Moreover, the addition of EGTA, a well-known Ca^{2+} chelator, restores the activity of TdCAT1 in presence of TdCaM1.3/Ca^{2+} (Fig. 7b), whereas EGTA alone does not alter the TdCAT1 activity. This result indicated that Ca^{2+} is necessary for the activation of TdCAT1 by the calmodulin TdCaM1.3. As Mn^{2+} and Fe^{2+} were shown to enhance the TdCAT1 catalytic activity (Fig. 2), we also evaluated the effects of TdCaM1.3/Ca^{2+} on the TdCAT1 activity in the presence of those cations. Remarkably, in a buffer containing 2 mM of Mn^{2+} or Fe^{2+}, addition of Ca^{2+} slightly increases the catalase activity of TdCAT1 (Fig. 8a). In the presence of TdCaM1.3/Ca^{2+} complex, addition of Mn^{2+}stimulates the activity of TdCAT1 (Fig. 8b). TdCaM1.3 and calcium rather increase TdCAT1 activity by about 2-fold comparatively to the activity measured only in the presence of CaM/Ca^{2+} alone (Fig. 8b). This stimulatory effect of TdCaM1.3 occurs albeit with lower efficiency even with concentrations of Mn^{2+}, Fe^{2+} and Ca^{2+} as low as 0.5 mM (Fig. 8b). In presence of Mn^{2+}, the increase reached it's maximum with 0.5 mM, while in presence of Fe^{2+}, the activity started to increase gradually and reached it's maximum in presence of 2 mM Fe^{2+} (Fig. 8c). This increase is calcium-dependent because addition of EGTA is sufficient to return the TdCAT1 activity to its initial level (Fig. 8b). Finally, to confirm the observed regulatory effects of TdCaMs/Ca^{2+} on TdCAT1 activity, we performed a new series of phosphatase assays using the truncated form His_$TdCAT_{200}$. As

expected, the activity of His_TdCAT$_{200}$remains unchanged in the presence of TdCaMs/Ca^{2+}. There is neither a negative (in the absence of Ca^{2+}; Fig. 9a) nor a positive effect (in the presence of TdCAT1/ TdCaM1.3 ratio molar of 1:4 and Ca^{2+}; Fig. 9b) of TdCaM1.3 on the catalytic activity of this truncated TdCAT$_{200}$ protein mainly in presence of increasing quantities of CaMs in the medium (data not shown). The same effect is observed in presence ofMn^{2+} and Fe^{2+} cations (Fig. 9c). All together, these data confirm that the catalytic activity of TdCAT1 can be specifically activated by CaM/Ca^{2+} in the presence of Mn^{2+} and Fe^{2+}.

4. Discussion

In plants, calcium is an important nutriment for growth and development such as thigmotropism, gravitropism, cell division and elongation [43]. Moreover, calcium plays an important role in plant resistance under various stress conditions including both biotic and abiotic stresses. In another hand, calcium is considered as an important second messenger in plants under various developmental cues, as well as under different stresses, including salinity stress [44]. Under normal condition, the resting cytosolic concentration of calcium, [Ca^{2+}]cyt, in plant cells is maintained at nanomolar level, mostly in the range of 10–200 nM, whereas the concentration of Ca^{2+} in cell wall, vacuole, endoplasmic reticulum and mitochondria is 1–10mM [45]. However, stress can trigger a sudden increase in the [Ca^{2+}]cyt level up to micromolar level [44]. The modification of calcium concentration in cells are perceived by several calcium sensors like, Calcineurin-B-like (CBL), Calmodulin (CaM) and Calmodulin-like (CML) proteins [42]. The most studied protein is CaM. This acidic protein is involved in many fundamental processes and stress responses. It has been suggested that CaM/Ca^{2+} complex was involved in the adjustment of cell metabolism, phytohormone signaling, ion transportation, and transcriptional regulation [19]. Ca^{2+}/CaM complex-mediates also plant response to different environmental stresses such as cold stress[47], drought stress [48], heat stress [49].

Plant catalases have been studied in many species such as Arabidopsis, sweet potato, pumpkin, and wheat [30, 32]. These proteins are involved in the detoxification ofH_2O_2into water and oxygen in all aerobic organisms. They are activated under developmental processes andin response to environmental stimuli [7]. In this work, the catalase activity of durum wheat TdCAT1 was analyzed. TdCAT1 had no activity at high acidic medium like CATs from rice (OsCAT-A, OsCAT-B and OsCAT-C), but it exhibits a maximum activity at pH 7 as shown for OsCAT-C [50] and for TaCAT1 from *Triticum aestivum* [51]. At25°C, those enzymes exhibit maximum activity, but it decreases gradually with the increase of temperature as shown for OsCAT-B [50]. Moreover, we revealed the presence ofMn^{2+} and Mg^{2+} binding domains. Those domains share the same characteristics with other known cations binding domains. Both metal cations are frequently bound by Aspartate (D) and Glutamate (E) residues situated in “β strand – random coil – β strand” and “β strand – random coil – α helix” structural motifs [52]. In association with Asp and Glu residues, Histidine residues are considered as major binder of Mn^{2+}cations [53].

Putative Ca^{2+} binding domains were also identified in the sequence of TdCAT1. Those domains have a degenerated EF-hand motif D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]. Ca^{2+} alone has a stimulatory effect on the catalase activity of TdCAT1.

Potential copper-interacting motifs were predicted and scored in Arabidopsis via copper-immobilized metal affinity chromatography (Cu-IMAC) [57]. Six candidate motifs, H-$(X)_5$-H, H-$(X)_7$-H, H-$(X)_{12}$-H, H-$(X)_6$-M, M-$(X)_7$-H, and H-$(X)_3$-C, are present in Cu-IMAC- isolated proteins with higher frequency than in the whole Arabidopsis proteome. Here, the sequence analyzes of TdCAT1 showed that the protein contains a H-$(X)_{12}$-H, binding motif type. Therefore, as far as we know, we demonstrate here for the first time that plant catalases harbors cations binding domains and those different bivalent cations such as Mn^{2+}, Mg^{2+} and Ca^{2+} can stimulate the activity of a plant catalase. An additional observation came from the deleted His-$TdCAT_{200}$ and His-$TdCAT_{295}$ forms (where the C-terminal part including the catalase domain and the catalase related immune-responsive were deleted). Those forms showed a decrease in catalase activity comparing with full length and His-$TdCAT_{460}$ form. Interestingly, those forms can be still enhanced by Ca^{2+} and Mn^{2+}. We can thus speculate that TdCAT1 could be activated in vivo but in presence of lower concentrations of cations. Interestingly, researches on the contribution of this cation in the modulation of catalase activity in vivo deserves further investigations.

In another part of this work, we identified a putative Calmodulin binding domains in the TdCAT1 sequence located at it's C-terminal part (459-482 aa). It was reported that this domain is essential for calmodulin binding and activation of some plant CATs in calcium dependent manner [30]. CaMs are Ca^{2+}-binding proteins found in all eukaryotes. Those proteins mediate the primary intracellularCa^{2+} signaling pathways. Elevation in Ca^{2+} concentration in nucleus or in the cytosol induces the formation of Ca^{2+}/CaM complexes which interact with an important number of targets such as ion transporters, protein kinases, Pathogen-Related proteins, transcription factors, and protein phosphatases and regulate cellular functions [22, 24, 26–27]. In plants, investigation of CAT proteins interaction with CaM/Ca^{2+} complex remains poorly investigated. In this study, we demonstrated that wheat catalase belonging to class I interacts in vitro with a wheat calmodulin (TdCaM1.3) as previously reported for other plant catalases such as in Arabidopsis and sweet potato. Interestingly, this interaction was calcium independent since TdCAT1 was able to bind to TdCaM1.3 in GST pull down assay mainly in absence of Calcium or in presence of the calcium chelator EGTA.

It is well known that CaM/Ca^{2+} complex can modulate the enzymatic activity of different proteins. Thus, we investigated the effect of CaM alone in the modulation of Catalase activity as we found that this interaction was Ca^{2+} independent (Fig. 6). In our work, CaM alone was not able to modulate this activity which increases by adding Ca^{2+} in presence of CaM. Such effect was previously shown for other plant catalases. Moreover, addition of Mn^{2+} cations to CaM/Ca^{2+} complex increases slightly the catalase activity of TdCAT1.The positive Mn^{2+}-mediated effect of CaM/Ca^{2+} requires a direct interaction between TdCAT1 and Mn^{2+} via the C-terminal part of the protein as the deleted form His-$TdCAT_{200}$. This was also

confirmed by investigating the catalytic activity of the truncated form His_TdCAT$_{200}$ in presence of TdCaM1.3/Ca^{2+} complex. In this case, the registered effect was noted in presence of Mn^{2+} and there is no effect of the CaM/Ca^{2+} complex. All together, these results suggest that Mn^{2+}cations act as a cofactor in the control of a plant catalase by the TdCaM1.3/ Ca^{2+} complex.

5. Conclusions

In conclusion, the data obtained in this study regarding TdCAT1 provide evidence for a novel regulatory mechanism where Calmodulin can modulate the catalytic activity of the wheat TdCAT1 in the presence or absence of Ca^{2+}.Interestingly, the catalytic activity is stimulated in the presence of CaM/Ca^{2+} complex. Moreover, the catalytic activity of TdCAT1 is modulated by Fe^{2+} and Mn^{2+} by a lesser extend in presence of Zn^{2+}, Cu^{2+} and Mg^{2+} in dose dependent manner. Further investigations should help to understand the contribution of such a regulatory mechanism in controlling plant responses to abiotic and/or biotic stresses.

Declarations

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Author contributions:

MG and FB have conceived and designed the work. MG, KF and ST performed all the experiments, MG and FB wrote the manuscript. NH helped in bioinformatic and statistical analysis. MH and FB have given the final shape of manuscript and all authors approved the manuscript.

Compliance with Ethical Standards

Conflict of interest:

The authors declare no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability:

All of the raw data will be made available on reasonable request to the corresponding author.

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Table

Table 1. list of primers used in PCR amplification of TdCAT1 and its truncated forms. The forward primer CF was used for the amplification of all forms with the use of reverse primers corresponding of each one.

Amplified fragments	Nucleotide sequences	Primers names
	TCGAATTCATGGACCCCTACAAGTA	CF
TdCAT1	ATCTCGAGTTACATGCTCGGCTTGG	CR1
$TdCAT_{200}$	ATCTCGAGGGCGGTAGTCGGCGGG	CR2
$TdCAT_{295}$	ATCTCGAGCCTCGGGCCACGTCTTG	CR3
$TdCAT_{460}$	ATCTCGAGGGTGAGGCGGGGGTCC	CR4

Figures

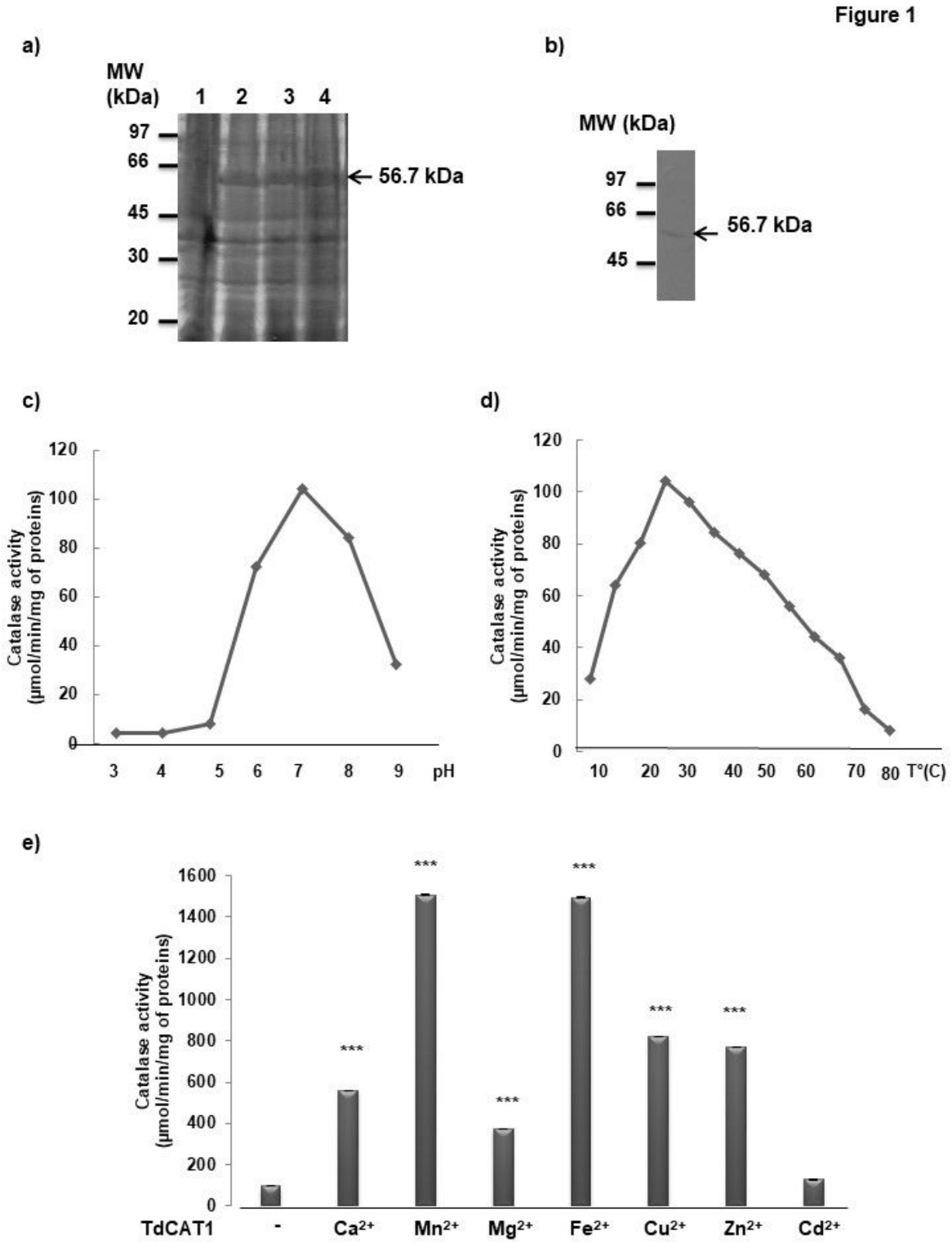

Figure 1

Functional characterization of TdCAT1. **(a)** Production of durum wheat recombinant His_TdCAT1 protein. Protein extracts from non-induced (lane1) and IPTG induced (lane2, 3 and 4) *E. coli* cells expressing pHis_TdCAT1 are presented. The lanes 2, 3 and 4 represent different incubation time in presence of 1 mM IPTG. **(b)** SDS-PAGE analyses of the purified recombinant proteins His_TdCAT1. Positions of the purified proteins are indicated by arrows. The size of protein standards is given in kDa on the left side. **(c)** Effect

of pH on the catalytic activity of TdCAT1. pH stability of the catalytic activity of TdCAT1 was studied in different pH medium ranging from 3-9 with a maximum activity at pH 7. **(d)** Effect of temperature on the catalytic activity of TdCAT1. The catalytic activity was studied in presence of different temperature with 5°C difference graduation and maximum activity at 25°C. **(e)** Stimulatory effects of divalent cations on the *in vitro* phosphatase activity of the recombinant His_TdCAT1. The catalytic activity is stimulated in the presence of 2 mM Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+} but not in presence of Cd^{2+}. TdCAT1 activity was assayed with 160 µg of recombinant Protein after incubation for 1 min in presence of 50mM H_2O_2 as a substrate in a 75 mM phosphate buffer at pH 7.0 and 25°C and the absorbance was measured at 240 nm. H_2O_2 decomposition was measured by measuring the decrease of H_2O_2 absorbance and was calculated as µmol H_2O_2 decomposed/mg protein/min. Values are means of initial rates (µmol/min/mg of proteins) ± S.E from at least three independent experiments with identical results.

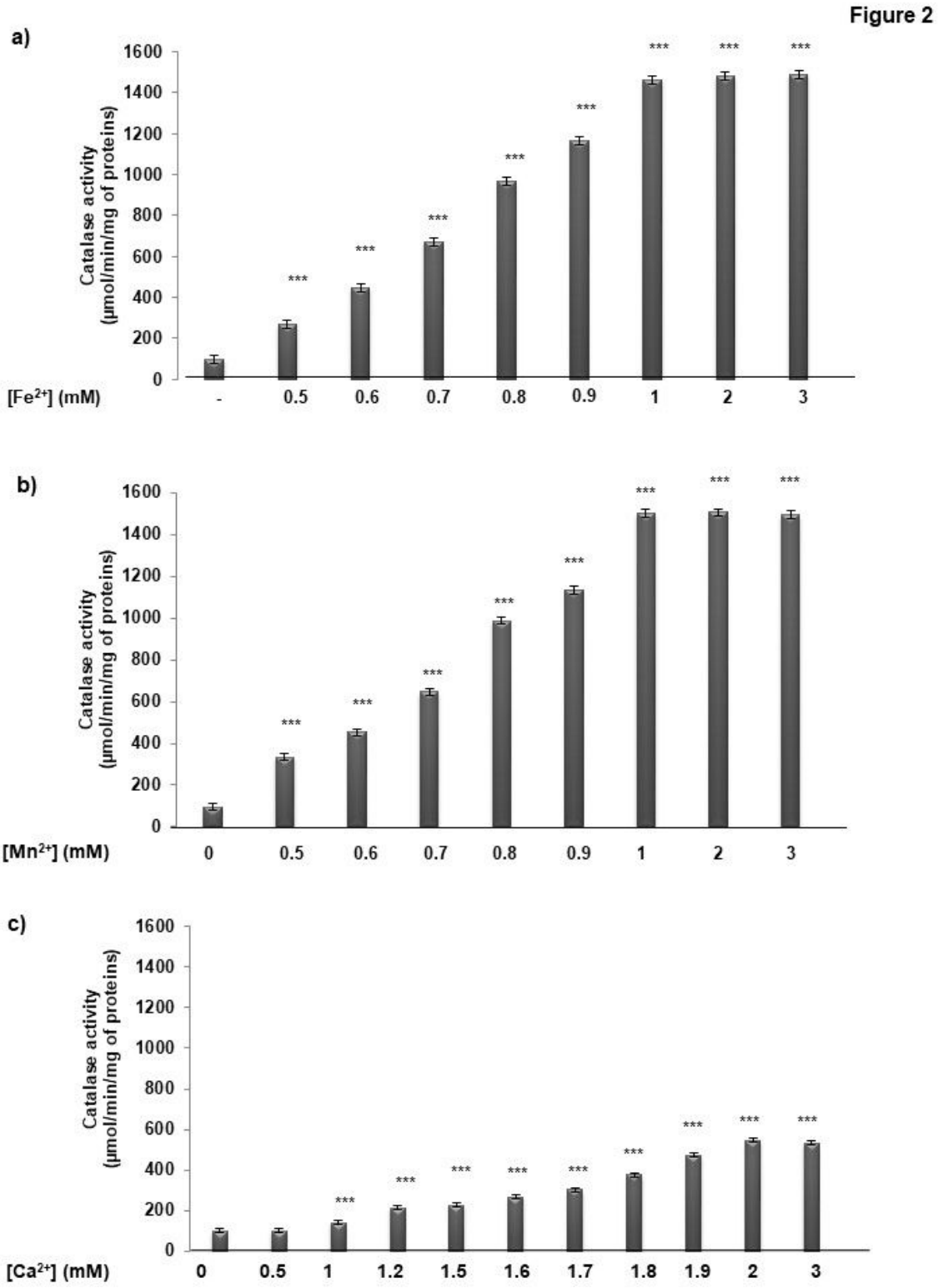

Figure 2

Stimulatory effects of Mn^{2+}, Fe^{2+} and Ca^{2+} on the *in vitro* catalase activity of the recombinant His-TdCAT1. TdCAT1 activity was assayed with 160 µg of recombinant His_TdCAT1 and 50 mM H_2O_2 as a substrate, in the presence of increasing concentrations of Fe^{2+} **(a)**, Mn^{2+} **(b)**, and Ca^{2+} **(c)**. Values are means of initial rates (µmol/min/mg of proteins) ± S.E from three independent experiments.

Figure 3

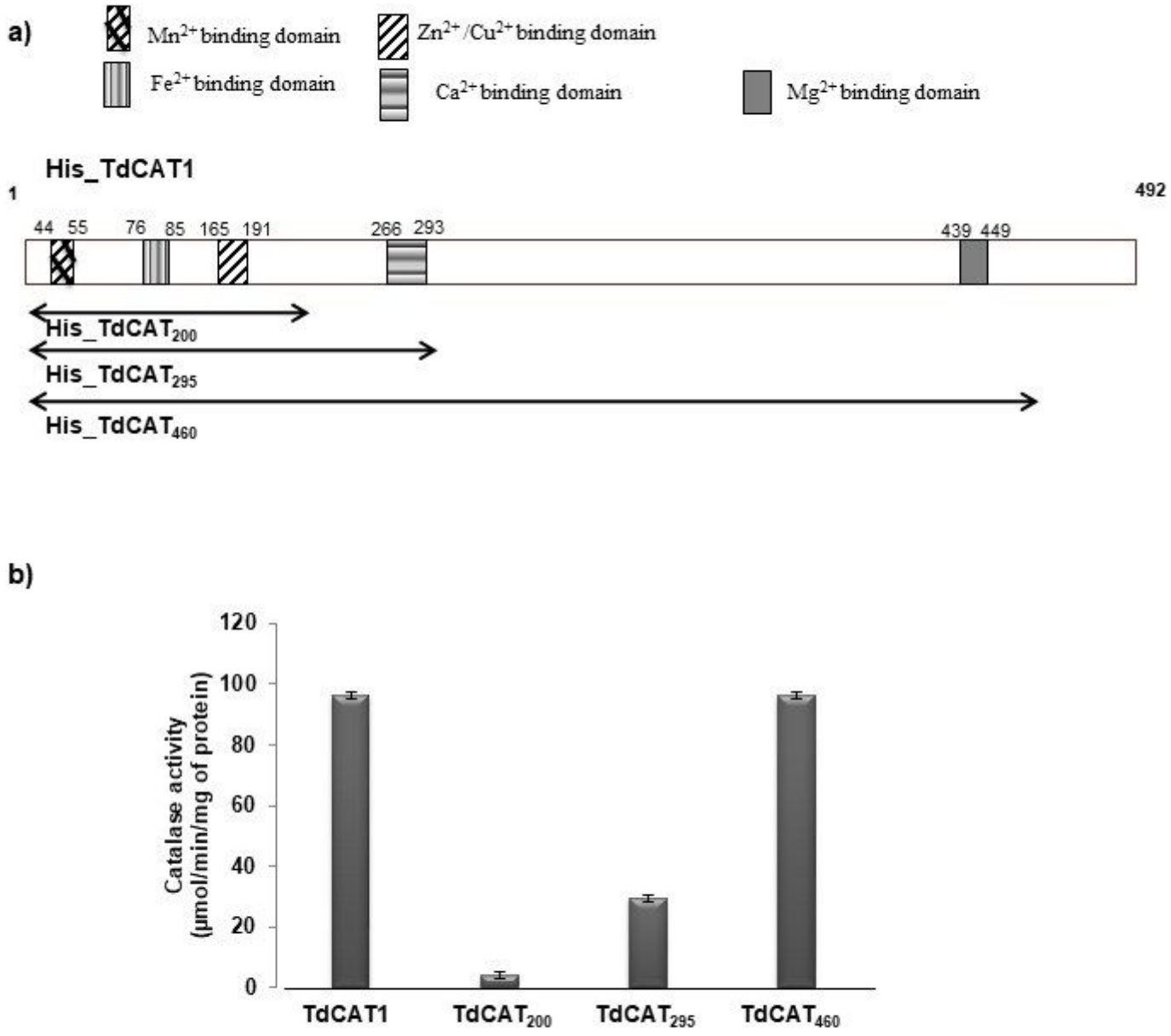

Figure 3

TdCAT1 harbors several putative cations binding domains located at different parts of the protein. **(a)** Schematic presentation of the full length TdCAT1 protein. The position of the different cation binding proteins is indicated. The conserved domains of TdCAT1 including the putative Calmodulin Binding Domain CBD and the putative Cation binding domains are presented by boxes with distinct patterns. **(b)** Schematic presentation of the different TdCAT1 isoforms used in catalase activity assays, TdCAT200 (1-200aa; b1); TdCAT295 (1-295; b2) and TdCAT460 (1-460; b3). The different domains are also represented on each form. **(c)** Catalase activity of the different recombinant proteins (160µg) in presence of 50 mM

H_2O_2 as a substrate. Values are means of initial rates (µmol/min/mg of proteins) ± S.E from three independent experiments.

Figure 4

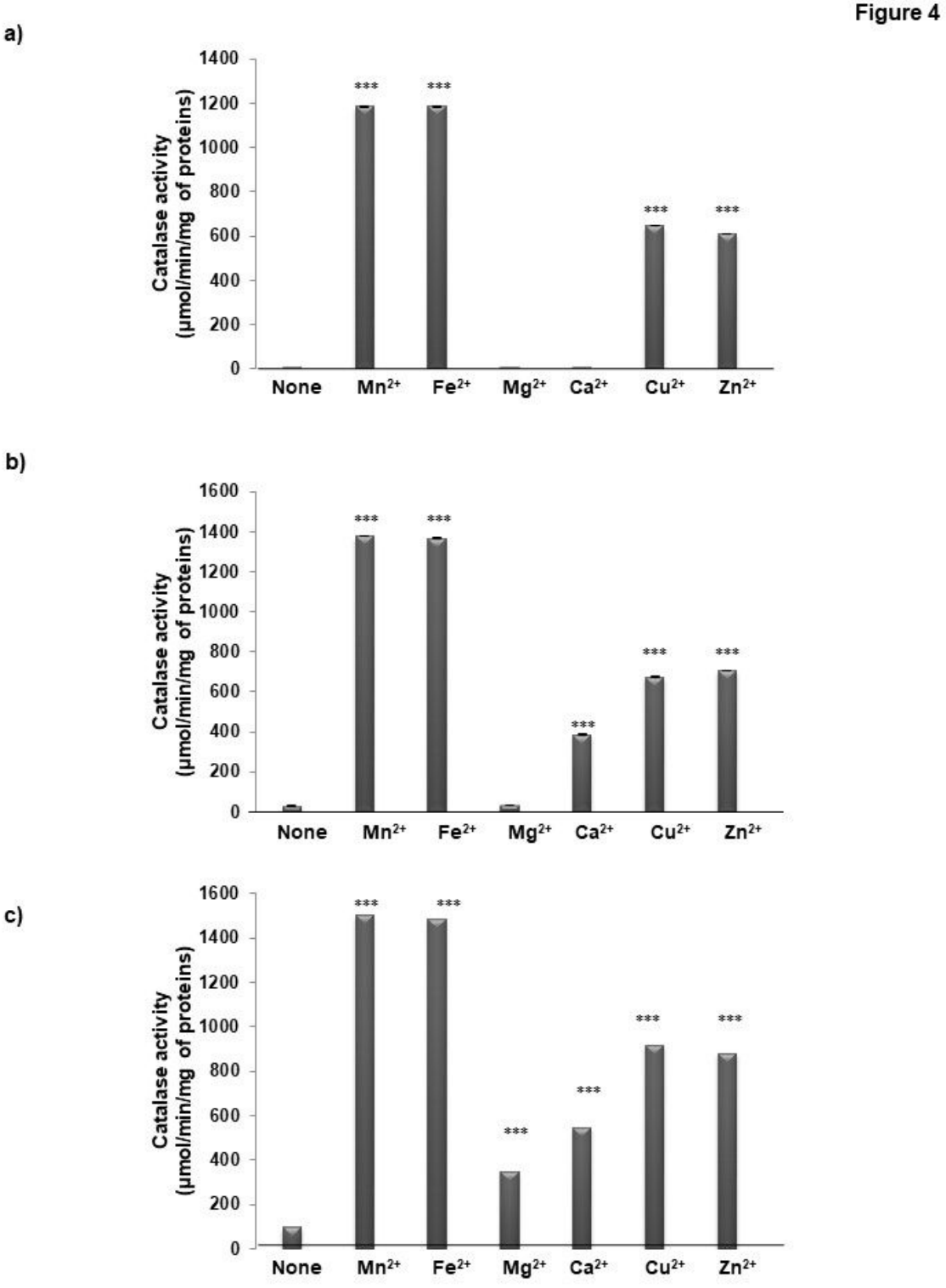

Figure 4

TdCAT1 harbors different cations binding domains located at different parts of the protein. Catalase activity of TdCAT200 **(a)**, TdCAT290 **(b)** and TdCAT460 **(c)** was assayed with 160 µg of recombinant

protein and 50 mM H_2O_2 as a substrate, in presence of 2mM of Mn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} or Zn^{2+}.Values are means of initial rates (µmol/min/mg of proteins) ± S.E from three independent experiments.

Figure 5

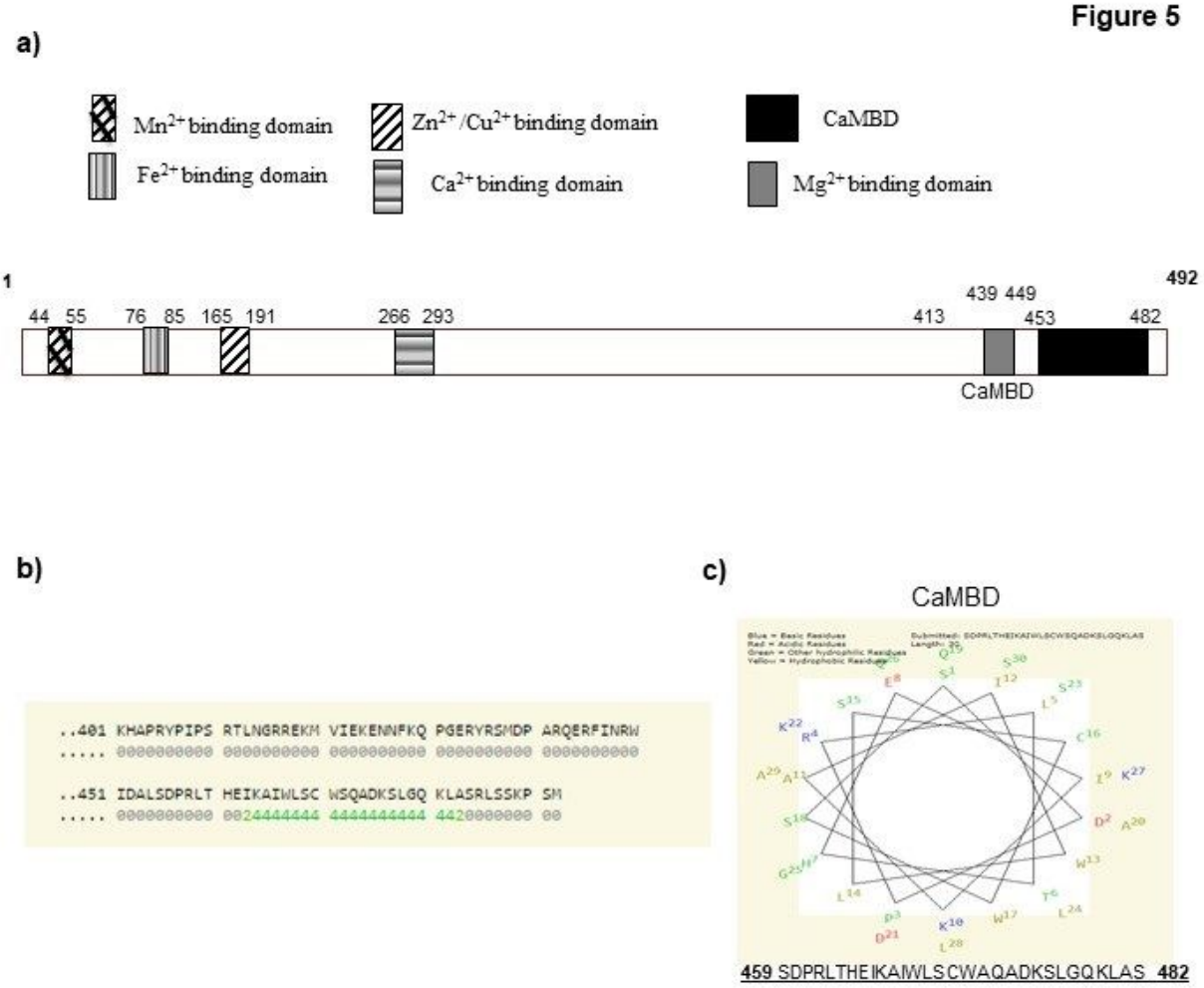

Figure 5

TdCAT1 harbors a conserved CaMBD located at the C-terminal part of the protein. **(a)** Schematic presentation of TdCAT1 with identification of the conserved CaMBD represented in black. **(b)** Helical wheel projection of the motif for the Calcium dependent calmodulin binding domain (underlined sequences). Dashed lines separate proposed hydrophobic (h) and basic (+) faces of the NT and CT wheels. **(c)** 3D structure of CaMBD as revealed by spdv 1.4 program. The basic amino acids are represented bin yellow, the hydrophobic amino acids are represented in pink.

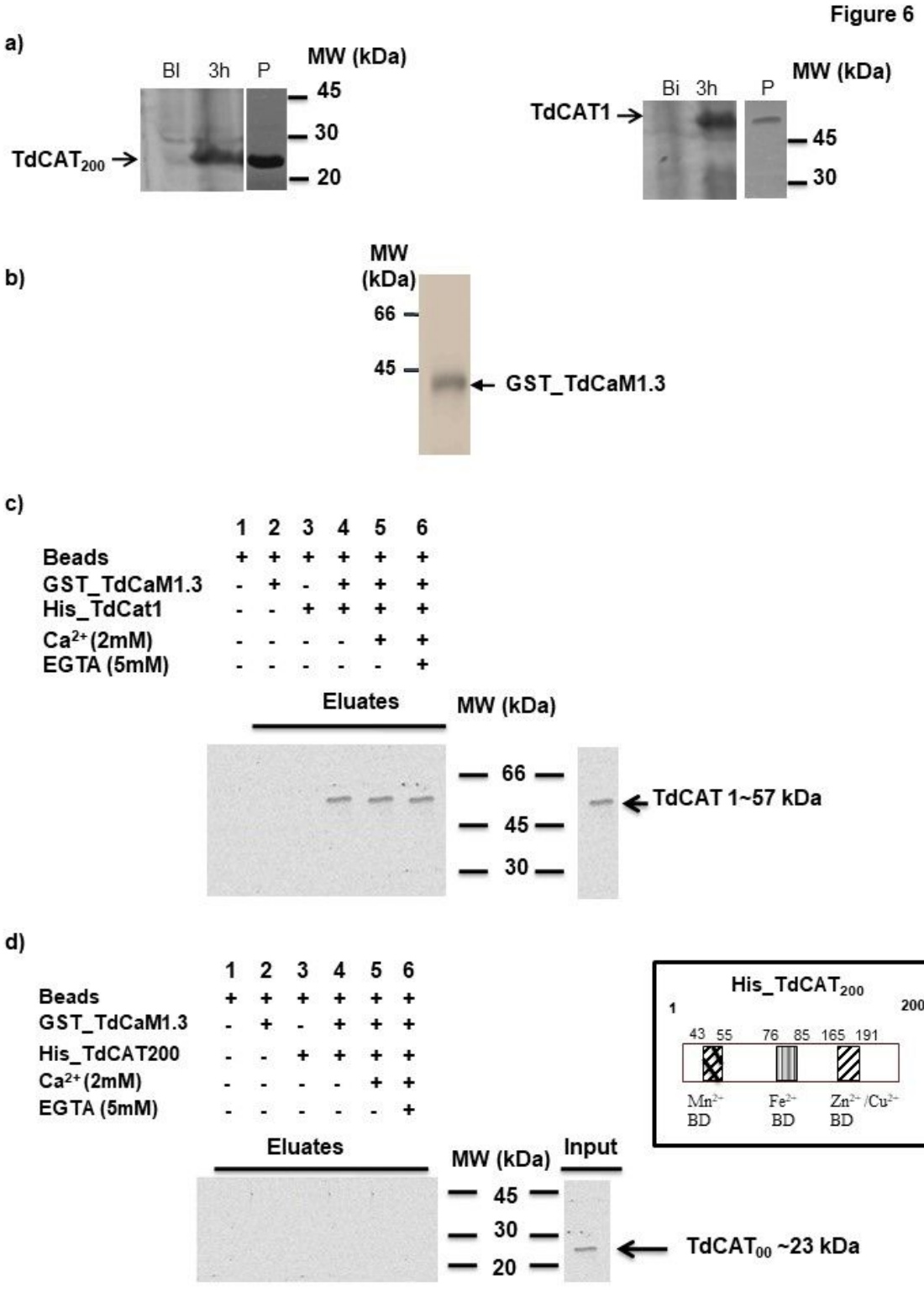

Figure 6

TdCaM1.3 interacts with type 1 Catalase proteins (TdCAT1). (**a)** Purification of recombinant proteins TdCAT1 and the deleted form TdCAT200. (**b)** Purification of recombinant GST-TdCaM1.3 proteins. (**c)** Physical interaction of TdCaM1.3 and TdCAT1 in vitro was verified by GST pull-down assay. GST-TdCaM1.3 was incubated in binding buffer containing glutathione-agarose beads with or without His_TdCAT1 and agarose beads were washed for five times and eluted. Lysis of *Escherichia coli (BL21)* (Input) and eluted proteins (Pull-down) from beads was immunoblotted using anti-His antibodies. **(d)** The deleted TdCAT200 form does not interact with GST-TdCaM1.3 by GST pull down assays as it was detected in the eluates.

Figure 7

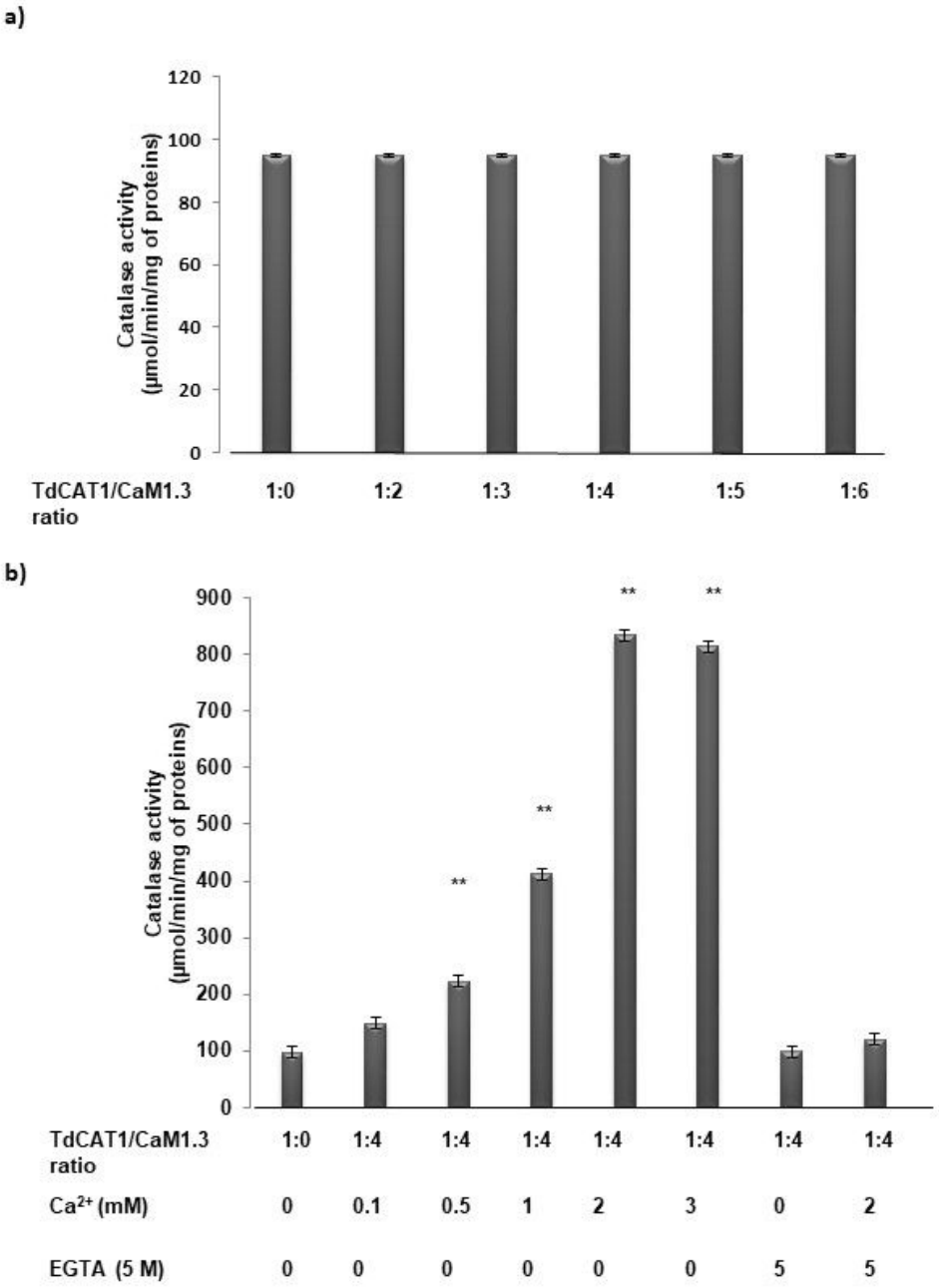

Figure 7

Effects of the TdCaM1.3/Ca^{2+} complex on TdCAT1 activity. **(a)** TdCaM1.3 alone has no effect on catalase activity of TdCAT1. **(b)** Stimulatory effect of the TdCaM1.3/Ca^{2+} complex in presence of increasing concentrations of Ca^{2+} from 0 to 3 mM and EGTA (5 mM) as indicated. Catalase assays were performed in the same buffer conditions as mentioned in presence of TdCAT1/tdCaM1.3 ratio molar 1:4. All data are mean values ± S.E of initial rate from three independent assays. (***) indicates value

significantly different from the control. Statistical significance was assessed by applying the ANOVA test with $\alpha<0.005$.

Figure 8

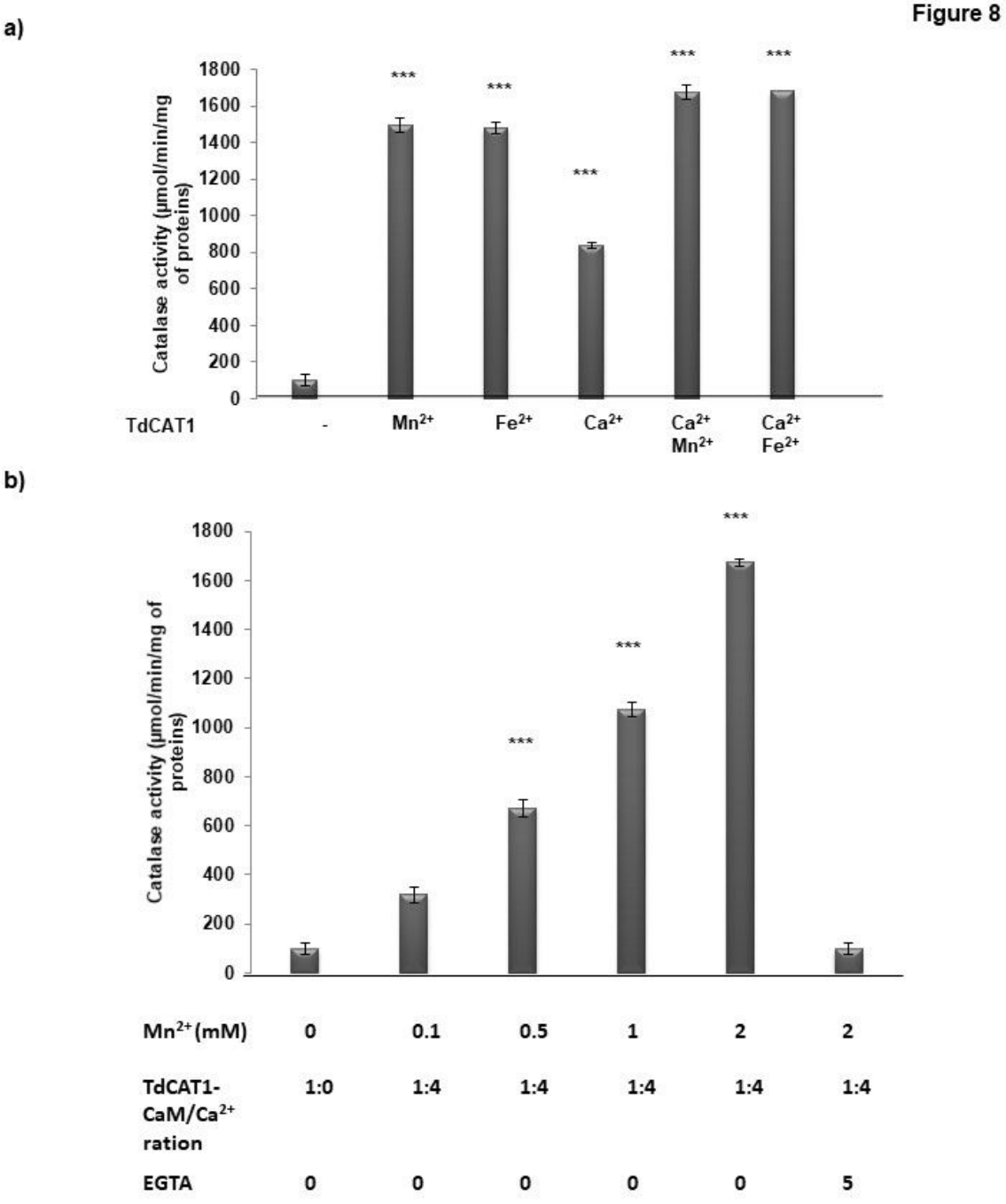

Figure 8

Effects of Mn^{2+} cations and CaM/Ca^{2+} complex on TdCAT1 activity. **(a)** Ca^{2+} cations further enhance catalase activity of TdCAT1 in presence of Mn^{2+} and Fe^{2+}. **(b)**Stimulatory effect of TdCaM1.3/Ca^{2+}

complex on His_TdCAT1 activity in the same buffer conditions using increasing concentrations of Mn^{2+} and in presence of TdCAT1/TdCaM1.3 ratio molar of 1:4. All data are mean values ± S.E of initial rate from three independent assays. (***) indicates value significantly different from the control. Statistical significance was assessed by applying the ANOVA test with $\alpha<0.005$.

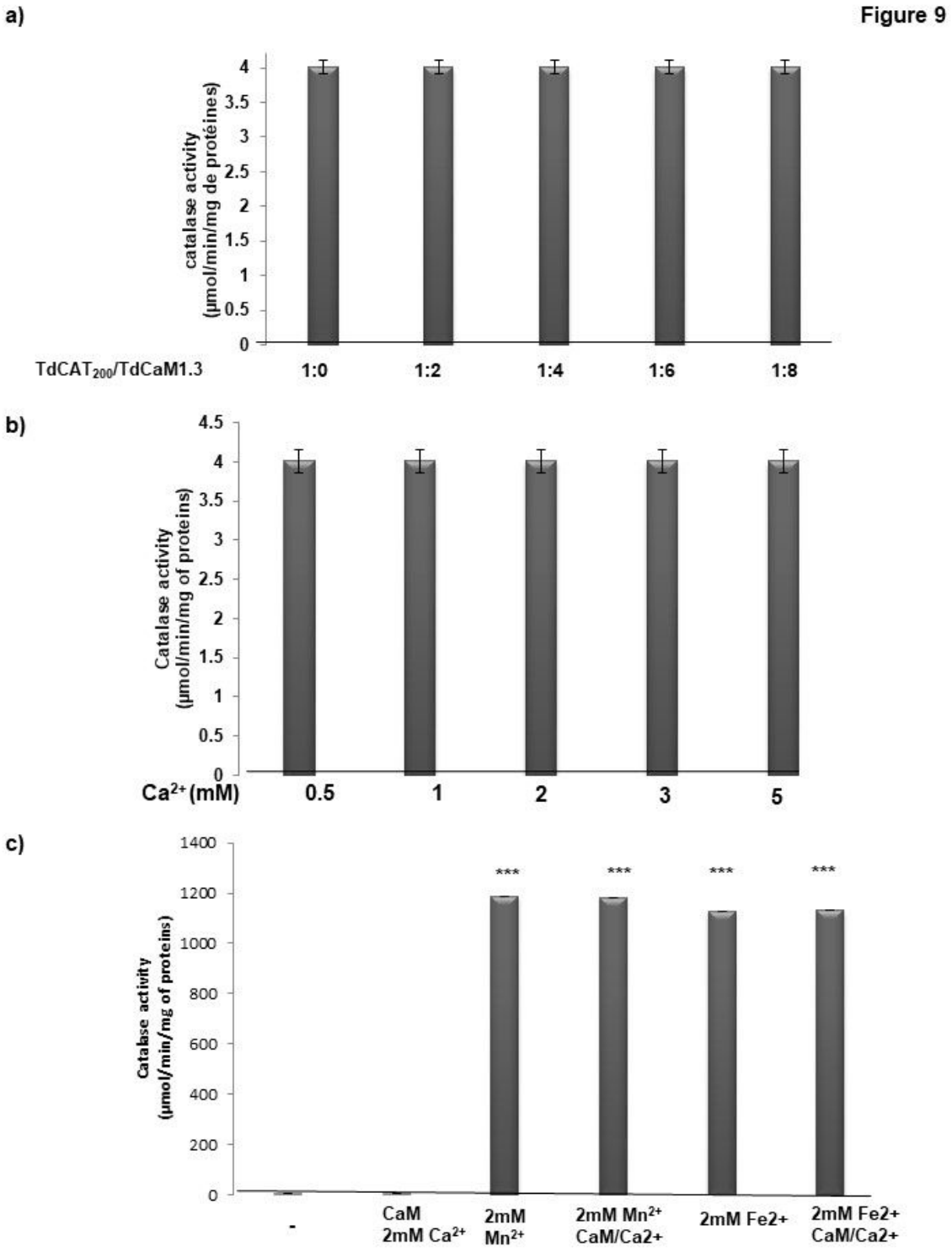

Figure 9

The activity of His_TdCAT1$_{200}$ is not affected by TdCaM1.3/Ca^{2+} complex. **(a)** Catalase activities were measured according to the same conditions indicated before with a His_TdCAT1$_{200}$/TdCaM1.3 molar ratio ranging from 1:0 to 1:8 1:4. **(b)** TdCAT1$_{200}$ activity was not modified in presence of increasing concentrations of Calcium ranging from 0.5 to 5 mM. **(c)** Catalase activity of deleted form His-TdCAT$_{200}$ was stimulated in presence of Mn^{2+} anf Fe^{2+} but not with CaM/Ca^{2+} complex. All data are mean values ± S.E of initial rate from three independent assays. (***) indicates value significantly different from the control. Statistical significance was assessed by applying the ANOVA test with $\alpha<0.005$.

Supplementary Files

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