Biochemistry and Biophysics Reports 4 (2015) 299–305

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Production, in *Pichia pastoris,* of a recombinant monomeric mapacalcine, a protein with anti-ischemic properties ☆

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form
2 October 2015
Accepted 6 October 2015
Available online 9 October 2015

Keywords:
Mapacalcine
Pichia pastoris
Calcium
Ischemia/reperfusion
Protein production

ABSTRACT

Mapacalcine is a small homodimeric protein of 19 kDa with 9 disulfide bridges extracted from the *Cliona vastifica* sponge (Red Sea). It selectively blocks a calcium current insensitive to most calcium blockers. Specific receptors for mapacalcine have been described in a variety of tissues such as brain, smooth muscle, liver, and kidney. Previous works achieved on hepatocytes and nervous cells demonstrated that this protein selectively blocks a calcium influx triggered by an ischemia/reperfusion (I/R) shock and efficiently protects cells from death after I/R. The aim of this work was to produce the recombinant mapacalcine in the yeast *Pichia pastoris*. Mass spectrometry, light scattering analysis and biological characterization demonstrated that the recombinant mapacalcine obtained was a monomeric form with 4 disulfide bridges which retains the biological activity of the natural protein.

1. Introduction

Marine sponges represent a source of molecules with therapeutic interests [1–4]. Limiting the extent of tissue damages after I/R injury is crucial for the success of organ functionality recovery. Once the blood flow restored, calcium homeostasis is completely disrupted. High levels of calcium activate biological events leading to cell death. Mechanisms underlying I/R injury are not yet clearly understood [5,6] and no treatment ensures a good recovery of organ functions after I/R damages. Mapacalcine is a homodimeric protein (MW 19 kDa; P86916), produced by a marine sponge (*Cliona vastifica*) [7]. Receptors for this protein have been detected in intestinal smooth muscle, brain, kidney, and liver [7–11]. Studies conducted on cultured hepatocytes demonstrated that mapacalcine inhibited a calcium influx triggered by I/R without affecting cell viability [10]. On mouse neurons, mapacalcine inhibited a calcium influx triggered by oxygen glucose deprivation (OGD), leading to neuron survival [12]. Mapacalcine and its receptor represent a starting point toward drug discovery for cell protection against I/R damages, and toward the understanding of a possible mechanism leading to cell death after an I/R shock. Further investigations to determine the minimal domain responsible for protein activity, together with the necessity to generate mutants, will require a stable source of protein. We describe here the production and purification of a monomeric recombinant form of mapacalcine from *Pichia pastoris* retaining the properties of natural mapacalcine.

2. Materials and methods

2.1. Production of recombinant 9HIS-mapacalcine in Pichia pastoris

A 270 nucleotide sequence deduced from the peptidic sequence [7] was designed according to the yeast codon usage for the production of a mapacalcine recombinant form (Fig. 1A) in *P. pastoris* X33 strain. The mapacalcine synthetic gene (Eurofins MWG/operon, USA) was cloned into a modified pPICZα vector carrying a 9 Histidine tag, a polyN spacer and the enterokinase (EK) and TEV protease cleavage sites fused to the N-terminal part of the secreted protein (Fig. 1B). The expression plasmid was linearized with the

☆This paper is dedicated to the memory of Doctor Bernard Gallois. His untimely death is a loss to our scientific community and we will greatly miss his insight, guidance, and friendship.

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http://dx.doi.org/10.1016/j.bbrep.2015.10.003

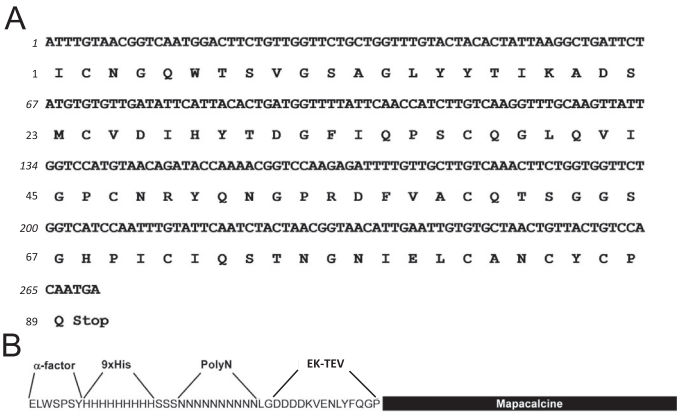

Fig. 1. Synthetic gene construction of mapacalcine from its aminoacid sequence. A: Mapacalcine monomer (89 aminoacids) and its deduced synthetic gene sequence (270 nucleotides). B: Map of the expressed 9HIS-mapacalcine cassette inserted in *Pichia pastoris* genome.

endonuclease DraI and inserted by electroporation into the yeast. The transformants were first selected on YPD-agar plates containing 0.2 mg/mL zeocin. Then, transformants carrying the highest number of integrated expression cassettes coupled with zeocin resistance were selected on YPD-agar plates by increasing the zeocin concentration up to 8 mg/mL.

Protein expression was assayed with several transformants. They were grown at 30 °C in 25 mL BMGH (100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.4 μg biotin, 1% glycerol) until culture $OD_{600}=2$. Cells were harvested, then expression induced in 50 mL BMMH (100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.4 μg biotin, 0.5% methanol) during 5–7 days at 30 °C, with addition of methanol (0.5% final concentration) every day. The secreted recombinant mapacalcine was detected by Western blot (WB) analysis with an anti His-tag antibody. The production was then scaled up in a 2 L fermentor with the transformant selected for best production of the recombinant protein. For convenience we call 9HIS-mapacalcine the recombinant ELWSPSY-9HIS-polyN-EK-TEV-mapacalcine obtained after secretion (see sequence in Fig. 1).

2.2. Large scale expression of recombinant 9HIS-mapacalcine.

Growth media were purchased from Becton Dickinson (Le Pont-de-Claix, France), buffers for chromatographic runs, and reagents were prepared using chemicals of analytical grade from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Transformed *P. pastoris* cells were grown in shake flasks containing 100 mL of buffered minimal glycerol BMGH with 150 μg/mL zeocin until optical density of 4–6. The culture was inoculated in 2 L of BMGH in BIOSTAT® Bplus laboratory fermentor (Sartorius-Stedim, Germany). The temperature and pH were maintained at 30 °C and 6.0, respectively. Dissolved oxygen levels were maintained at 15% saturation by regulating aeration and agitation in a cascading system. After complete consumption of glycerol in the medium corresponding to 24 h batch growth phase, a methanol fed-batch phase was initiated by adding methanol every 8 h to a final concentration of 0.6%. The frequency of the methanol addition was calculated to maintain the dissolved oxygen level around 15% during the induction phase. After 120 h of induction, the culture was harvested. The cell-free broth pH was adjusted to 7.4 and the mixture was filtered on 0.45 μm cellulose acetate membrane (Sartorius-Stedim) and frozen at −80 °C.

2.3. Purification of recombinant 9HIS-mapacalcine.

IMAC (Ni-NTA) purification experiments were optimized on HisTrap FF crude 1 mL pre-packed column (GE Healthcare, Sweden). The HisTrap™excel 5 mL ready-to-use IMAC columns were used for the higher scale purification. The Ni-NTA column was equilibrated with 50 mM Tris–HCl pH 7.4, 500 mM NaCl. The culture medium was loaded onto the column. Elution was carried out using 50 mM Tris–HCl pH 7.4, 500 mM NaCl, 500 mM imidazole. Binding and elution were performed at a flow rate of 3 mL/min.

The eluted fraction was dialyzed (Spectrapor 6 membrane MW cutoff 3500) against a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM ammonium acetate, lyophilized and re-suspended in 5 mL of water before being loaded on a HiLoad 16/600 Superdex 75 pg size exclusion chromatography column (SEC), previously calibrated with a gel filtration markers kit, 6500–66,000 Da. (Sigma-Aldrich). Elution was achieved with the dialysis buffer at 1 mL/min and monitored by $A^{280\ nm}$ recording.

Fraction P1 obtained from SEC was submitted to a reverse phase chromatography (Merck, LiChrospher® 100 RP-8, 10×250 mM, 10 μm). The column was equilibrated with H_2O containing 0.1 % TFA, and eluted at 3 mL/min with a gradient of acetonitrile (0.08% TFA) from 0% to 60% during 40 min. Elution was monitored by $A^{280\ nm}$ recording.

2.4. Enzymatic removal of the ELWSPSY-9HIS-polyN peptide.

6HIS-TEV protease (in-house produced) or EK (Sigma, Saint-Quentin-Fallavier, France) was used to remove the ELWSPSY-9HIS-polyN peptidic part of recombinant mapacalcine. For TEV protease cleavage, 9HIS-mapacalcine (10 mg) was incubated overnight at room temperature with 1 mg of enzyme in 50 mM Tris–HCl pH 7.4, 0.5 M urea and 0.25 mM 2-mercaptoethanol. For EK cleavage, enzyme (1 mg) and 9HIS-mapacalcine (10 mg) were incubated overnight at 30 °C in 100 mM Tris–HCl pH 7.4, 1 M urea, 2 mM $CaCl_2$ and 100 mM NaCl. The reaction mixture was loaded on an IMAC column (Ni-NTA) of 1 mL equilibrated with 50 mM Tris–HCl pH 7.4, 2 mM imidazole. The non-retained material supposed to contain the ELWSPSY-9HIS-polyN free recombinant mapacalcine was collected. The material retained on the column was eluted with the equilibration buffer supplemented with 500 mM imidazole. Fractions were analyzed by SDS PAGE and WB.

2.5. SDS PAGE and WB analyses.

SDS PAGE analyses were performed on 4–20% gels stained with colloidal Coomassie blue (BioRad). For WB, proteins separated on SDS PAGE were transferred during 1 h on a PVDF membrane (Immobilon P, Millipore), blocked during 1 h in PBS containing 0.1% Tween 20 (PBST), 2.5% BSA (PBST/BSA) and probed (2 h at room temperature or overnight at 4 °C) with an anti-penta-HIS antibody (Qiagen) at 1/3000 dilution in PBST/BSA. The blot was washed 6 times during 5 min with PBST, then incubated for 2 h at room temperature with an anti-mouse HRP conjugated secondary antibody (BioRad) at 1/3000 dilution in PBST/BSA. After 6 washes in PBST, the blot was revealed using the ECL system (Luminata® forte, Millipore).

2.6. Biological validation of recombinant mapacalcine.

Electrophysiological current recording, Oxygen Glucose Deprivation (OGD) and cell calcium imaging on mouse cortical neurons were achieved as described in [12] Controls were performed with the buffer in which proteins were dissolved.

2.7. Mass spectrometry analyses (MS)

Reduction of disulfide bonds (1 h at 55 °C) was achieved with 50 mM Dithiothreitol (DTT) in 10 mM ammonium acetate and alkylation (1 h at room temperature into the dark) with iodoacetamide (IAA) at 100 mM in 10 mM ammonium acetate (final concentrations). MALDI-MS analyses were carried out with a Ultraflex III mass spectrometer (Bruker) using sinapinic acid (Sigma-Aldrich) as a matrix (10 mg/mL solution in 50% acetonitrile/0.1% TFA) and a mixture of three proteins (Insulin $[M+H]^{+}_{average}=5734.52$ Da, Cytochrome C $[M+H]^{+}_{average}=12{,}360.97$ Da, Myoglobin $[M+H]^{+}_{average}=16{,}952.31$ Da) for external calibration. ESI-MS analyses were carried out using a Q-TOF Micro mass spectrometer (Waters). Accurate mass analysis was obtained by means of multiply charged ions of recombinant mapacalcine with MaxEnt (Waters) used as deconvolution software.

2.8. Light scattering analysis

A solution of recombinant mapacalcine (5 mg/mL) in 50 mM Tris–HCl pH 7.4, 150 mM ammonium acetate was submitted to light scattering analysis (LSA). The estimated molecular weight is expressed as mean ± standard deviation.

3. Results

3.1. Expression of 9HIS-mapacalcine in Pichia pastoris.

In large scale experiments, no expression was observed before addition of methanol (Fig. S1). Three days after adding methanol, a band corresponding to the expected molecular weight for the mapacalcine monomer appeared and remained present 5 days after induction.

3.2. Purification of 9HIS-mapacalcine

A large peak was eluted with 0.5 M imidazole from the IMAC column (Fig. S2). WB and Coomassie analyses showed that a weak band of less than 15 kDa was already detectable in the crude culture medium. Analysis of the flow-through demonstrated that 9HIS-mapacalcine was totally retained. No mapacalcine was eluted during the wash step. Peak P eluted between 500 and 520 mL corresponded to mapacalcine and very few mapacalcine was detected in the peak shoulder Sh (520–540 mL).

The eluted material was loaded on the SEC column. Different pools, P1–P4, were collected (Fig. 2A), analyzed by SDS PAGE and WB (Fig. 2B). Coomassie staining of the SDS PAGE demonstrated that P1 contained a single band corresponding to a strong signal in WB with a MW around 15,000. Coomassie staining of P2 exhibited a first band at a MW around 15,000 (close to the expected mass for 9HIS-mapacalcine) and a second one at a lower MW. The presence of this larger band accounts for the discrepancy observed between the signal in WB and the P1 and P2 optical densities. Fractions P3 and P4 did not exhibit bands in the Coomassie tracks and a very faint or no signal in WB respectively. Fraction P1, corresponding to a theoretical MW of 17 kDa according to column calibration, was selected and submitted to C8 reverse phase purification. The chromatogram (Fig. 2C) showed a single peak, suggesting that the purity of this fraction was suitable for further characterization and biological assays.

3.3. Molecular weight determination by LSA

The Molecular weight of the 9HIS-mapacalcine was determined by LSA which is a non-denaturing technology. Fig. S3 shows a typical curve obtained when a sample of recombinant mapacalcine is submitted to light scattering analysis. Data obtained exhibited a MW of 16.19 ± 1.28 kDa ($n=32$) which is in good agreement with the molecular weight (17 kDa) theoretically determined as a function of its elution volume from the SEC.

3.4. Removal of the ELWSPSY-9HIS-polyN peptide from mapacalcine.

After enzymatic cleavage and IMAC purification of the reaction mixture, SDS PAGE analysis of the flow-through and eluted fractions demonstrated that, regardless the enzyme used, no untagged mapacalcine was eluted suggesting that the ELWSPSY-9HIS-PolyN peptide was not removed (not shown). Therefore, we used 9HIS-mapacalcine for subsequent experiments.

3.5. Mass spectrometry analyses

Mass spectrometry studies were performed to assess the integrity

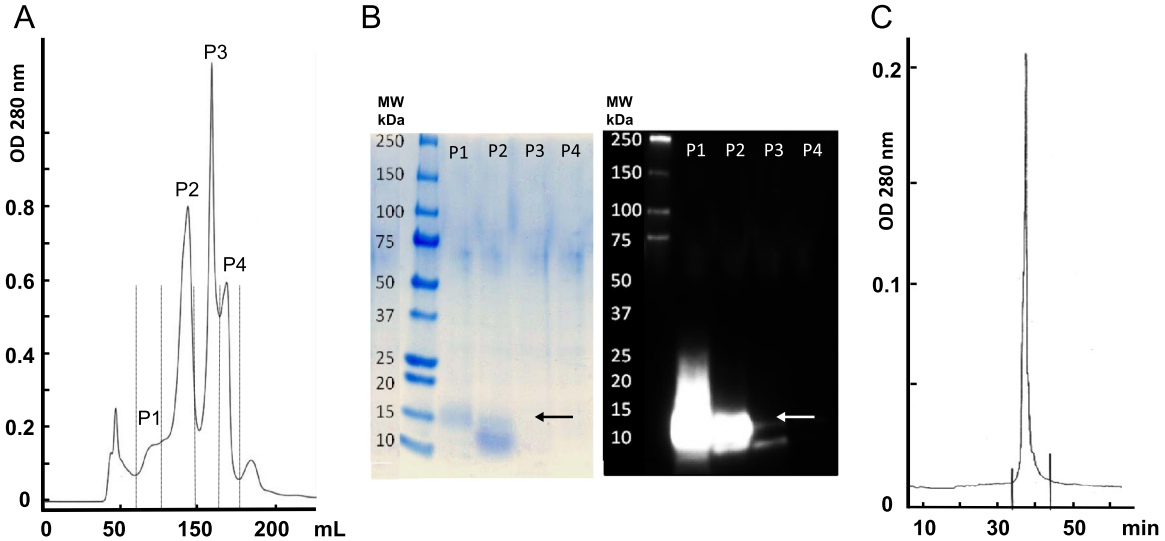

Fig. 2. Purification steps of 9HIS-mapacalcine. A: Elution profile (280 nm) obtained during the size exclusion chromatography step. Vertical lines delimitate the different pools. B: SDS PAGE (Coomassie staining) and WB analyses of pools P1–P4. C: Chromatogram (280 nm) obtained after injection of pool P1 on the C8 reverse phase column. Vertical lines indicate the recombinant mapacalcine.

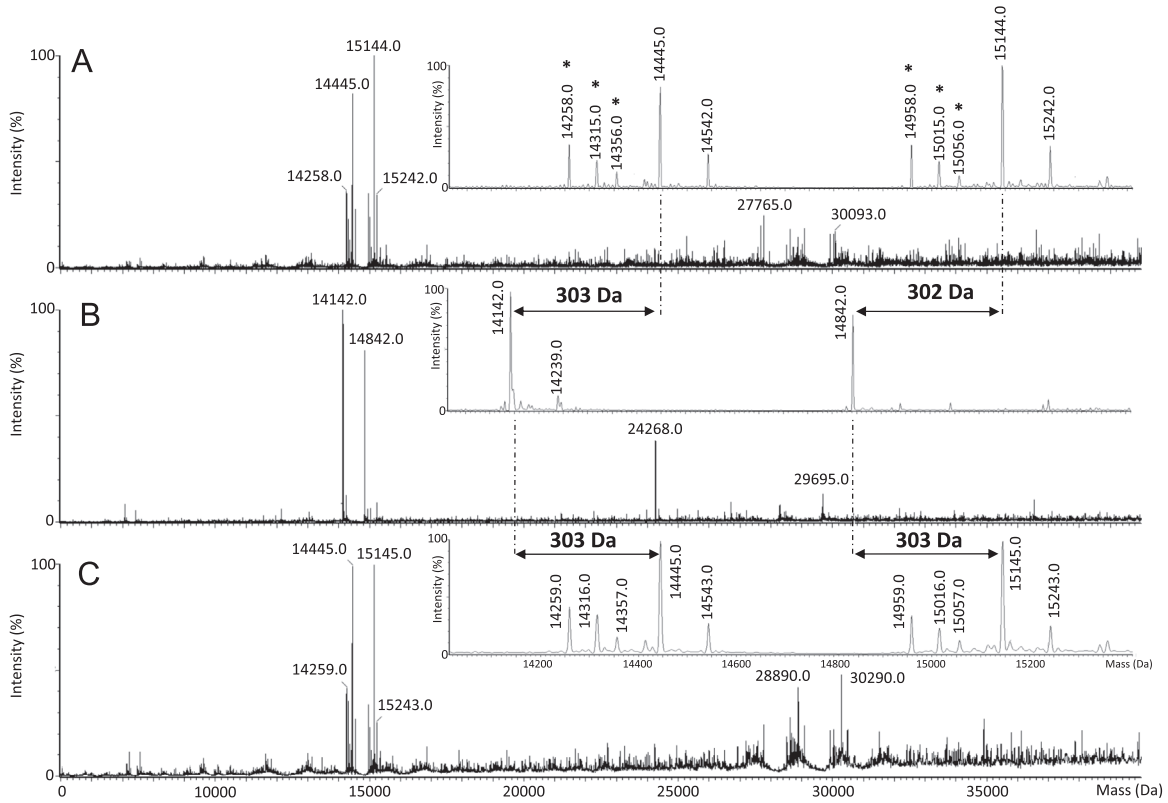

Fig. 3. ESI-MS analysis of 9HIS-mapacalcine. Deconvoluted spectra and zooms on regions of interest. 9HIS-mapacalcine was analyzed after different treatments: A: Untreated. B: treated with DTT. C: treated with iodoacetamide.

of the produced 9HIS-mapacalcine, to determine its oligomeric state and to evaluate the number of disulfide bonds. Electrospray ionization (ESI)-MS demonstrated the presence of two major forms at 14,445 and 15,144 Da (Fig. 3A). None of them corresponded to the theoretical mass of the recombinant monomeric protein calculated from the sequence (14,848 Da, taking into account all disulfide bonds in reduced form).

When a reducing treatment with DTT was applied, the mass of the two major peaks shifted to $[M+H]^+$ values of 14,142 and 14,842 Da (Fig. 3B). The highest mass matched the expected theoretical mass of a mapacalcine monomer bearing three to four residual disulfide bonds (among the four possible ones). These results are in good agreement with earlier observations on native mapacalcine indicating that disulfide bonds are difficult to reduce (unpublished data).

With or without reduction step, the mass difference between both peaks was unchanged (700 ± 1 Da). In each experiment, the lower mass was consistent with the elimination of the six first N-terminal amino acids (ELWSPS). Such an event is commonly encountered for heterologous expression of recombinant proteins in *P. pastoris* [13,14]. The mass shift observed for each peak after reduction (302 and 303 Da) was identical, within the mass tolerance, indicating that these two forms bore the same modification, probably a glutathione. Four satellite peaks were detected for each form (Fig. 3A). Peaks at respectively 14,542 and 15,242 Da exhibiting a mass difference of +98 Da (± 2) may correspond to a phosphate adduct. The three others, (black stars), cannot be explained by protein cleavage. As they disappear after treatment with DTT (Fig. 3B) they may correspond to uncharacterized adducts to a cysteine. Moreover, these additional forms cannot be due to deconvolution errors of ESI-MS spectra as at least two of them are detected by MALDI-MS analysis (Fig. S4).

Since no representative peak corresponding to a dimer was found by the software used to deconvoluate the ESI-MS spectra (Fig. 3A), these data suggested that the recombinant mapacalcine was a monomer. This result was confirmed by MALDI-MS which clearly showed the absence of dimer in the appropriate 29–30 kDa mass range (Fig. S4A).

Detection of possible free cysteines was achieved by IAA treatment, used as protein alkylating agent, followed by ESI-MS. No mass shift was observed (Fig. 3C), indicating that all cysteine sulfhydryl functions were blocked, suggesting that four disulfide bonds are formed and that the ninth cysteine does not react.

The IAA treatment, conducted on the reduced protein, led to two additional major peaks at 14,899 Da and 14,199 Da (data not shown), each showing a mass difference of 57 Da compared to the full-length mapacalcine and its degradation product (14,842 Da and 14,142 Da, respectively). This mass shift (in agreement with the binding of one unique IAA) demonstrates that the DTT treatment only eliminated the 302 ± 1 Da modification bore by one cysteine. Nevertheless, the presence of minor peaks observed for each protein (at +114 Da, +171 Da etc.) indicated that species with more than one IAA were present suggesting that disulfide bonds can be partially cleaved by the reducing treatment.

3.6. Protective effects of 9HIS-mapacalcine against hypoxia

We studied the protective role of 9HIS-mapacalcine using the OGD protocol, which is considered as the best reliable *in vitro* model for ischemia [15,16]. Effects of 9HIS-mapacalcine were compared to those of the native mapacalcine. OGD consists in a glucose and oxygen deprivation, 1.2% instead of 5% O_2 in normal conditions. Ten to twelve days aged mouse cortical neurons were submitted to OGD in the presence or absence of 1 μM 9HIS-mapacalcine or 1 μM native mapacalcine. After 2 h of OGD, the number of surviving cells was counted in nine 0.38 mm^2 fields randomly selected by a computer in each of six Petri dishes. It clearly appeared that 9HIS-mapacalcine as well as native mapacalcine increased cell survival

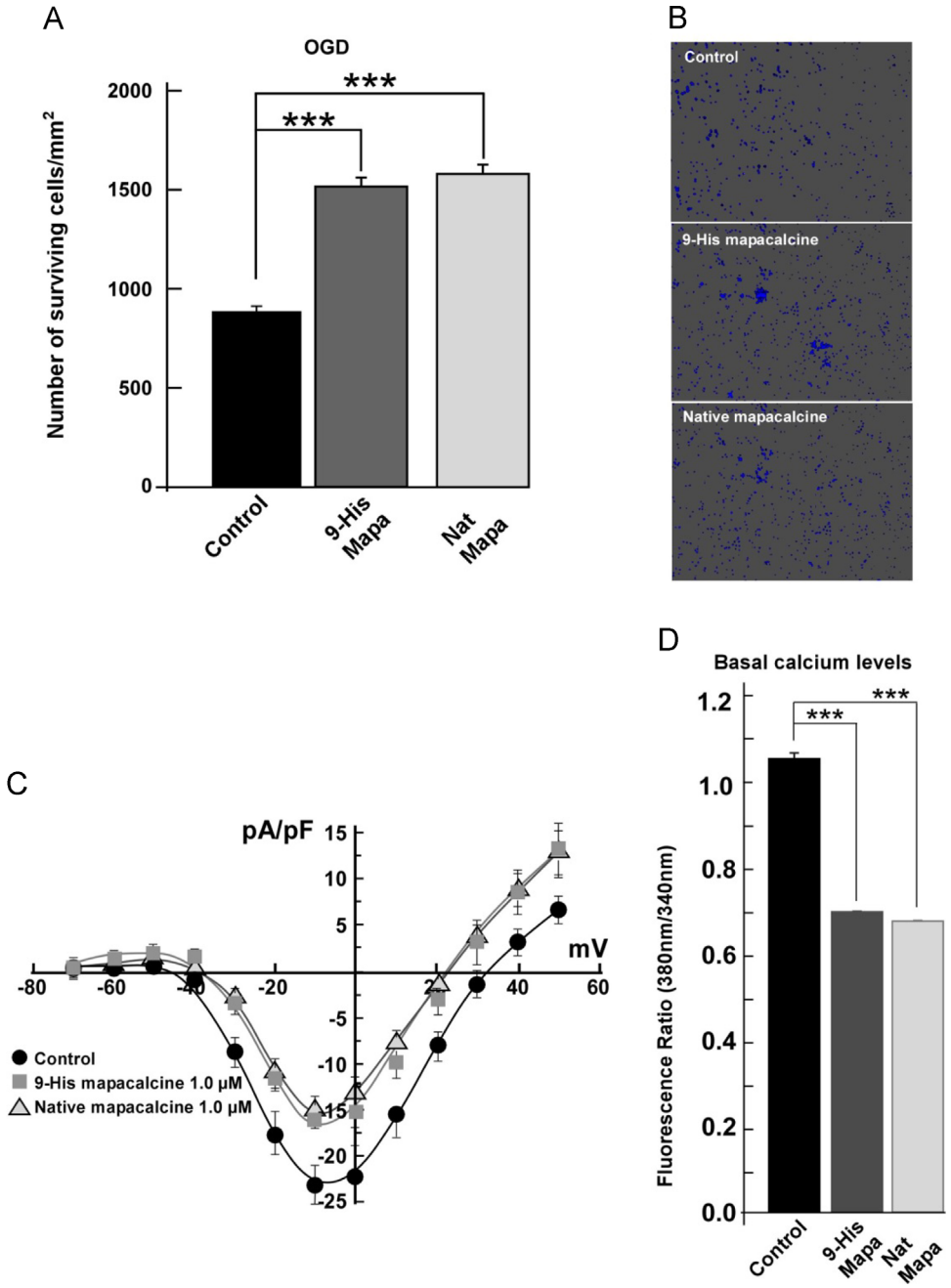

Fig. 4. Effects of 9HIS-mapacalcine and native mapacalcine on cortical neurons during OGD. A: Results were expressed as the mean value of cells per mm^2 ± SEM. Incubations were performed in the absence (Control) or in the presence of 1μM of 9HIS-mapacalcine (9-His mapa), or 1 μm of native mapacalcine (Nat mapa), $^{***}p < 0.005$ (Student *t*-test). B: Typical pictures of one counted field in control, 9HIS-mapacalcine and native mapacalcine conditions. C: Comparison of effects of 1 μM of 9HIS-mapacalcine or native mapacalcine on electrophysiological recordings in cortical neurons. The holding potential was −80 mV. Calcium currents were recorded from −70 to +50 mV in control condition (•), in the presence of 9HIS-mapacalcine (■) or native mapacalcine (△). In each condition, current densities (pA/pF) are shown as a function of membrane potential (mV). Bars represent the SEM values, $n = 10$ for each condition, $p < 0.05$. D: Basal calcium levels. The fluorescence intensity of a calcium indicator Fluo-2/AM was measured in the absence (Control) or in the presence of 1 μM of 9HIS-mapacalcine (9-His mapa) or native mapacalcine (Nat mapa). Results are means ± SEM of 6 independent dishes, $^{***}p < 0.005$ (Student *t*-test).

(1492 ± 22 and 1523 ± 35 versus 1091 ± 66 surviving cells/mm^2) for 9HIS-mapacalcine and native mapacalcine versus control, respectively (Fig. 4A and B). These results showed that 9HIS-mapacalcine acts as a protective agent against ischemia. This protective effect is strengthened by the fact that 9HIS-mapacalcine (1 μM), as native mapacalcine, was able to block a calcium current recorded on cortical neurons (Fig. 4C) and to reduce the calcium influx in basal conditions (Fig. 4D). The increase of intracellular calcium concentration is a major event that contributes to neuron death following an ischemic insult [17]. Taken together, these data evidence that the monomeric 9HIS-mapacalcine retains the same properties as the native mapacalcine [12].

4. Discussion

Disulfide bonds are a typical feature of secreted proteins. They often play a crucial role in the folding leading to the functionally active form of a protein. As the difficulty to reach the native conformation increases with the number of cysteine residues yielding

a number of possible isoforms, various organisms and expression systems have been used or designed to successfully produce disulfide-rich proteins. For that purpose, engineered strains like Origami or Shuffle have been developed in *Escherichia coli*, and *in vitro* expression systems tested [18–20]. As far as we know, the protein containing the highest number of disulfide bridges expressed in *E. coli* is resistin (11 cysteine residues, 108 aminoacids) [21]. Nevertheless, using such expression systems, we only obtained an insoluble form of recombinant mapacalcine suggesting that it was misfolded. Many proteins containing intra- or intermolecular disulfide bridges have been successfully expressed in *P. pastoris* [22,23], such as *Pisum sativum* defensin (8 cysteines, 47 aminoacids) [24], human α-defensin 5 (6 cysteines, 32 aminoacids) [25], and human hepcidin-25 (8 cysteines, 25 aminoacids) [26]. The highest cysteine containing protein expressed in *P. pastoris* is the secretory leukocyte protease inhibitor (16 cysteines, 107 aminoacids) [27]. We tried thus to express mapacalcine in *P. pastoris*. Data presented here demonstrate that *P. pastoris* produces a soluble and biologically active recombinant mapacalcine in reasonable amounts (5 mg of protein/L of culture medium).

Neither TEV protease nor EK was able to cleave the N-terminal part of the recombinant mapacalcine. This observation suggests that the enzyme cleavage site is probably embedded in the molecule while the 9HIS tag (or part of it), located 15 aminoacids farther toward the N-terminus of the molecule (see Fig. 1B), can interact with the Ni-NTA column. In all biological assays the presence of the ELWSPSY-9HIS-polyN-EK-TEV peptide did not modify the biological activity of the recombinant mapacalcine with respect to the activity of native mapacalcine suggesting that it does not bear residual activity. Whereas chromatographic data obtained after the C8 purification step (Fig. 2C) only demonstrated a single peak, mass spectrometry analyses revealed the presence of two 9HIS-mapacalcine monomeric forms. A possible explanation is that mass spectrometry only provides qualitative but not quantitative results. However the deleted aminoacids which characterized one form with respect to the other cannot be easily protonated. Thus, it is unlikely that such a modification alters significantly the ionization of each species and consequently the respective intensity of their peaks. Another explanation would be that these aminoacids poorly interfere with the C8 column so that they might have no noticeable impact on both species retention time.

We cannot determine if both forms are biologically active as we were unable to separate them. Nevertheless, several points are to be considered. Firstly, the 6 aminoacids deleted at the N-terminal position do not belong to the sequence of the natural mapacalcine. Moreover, these aminoacids are located 44 residues before the native mapacalcine sequence and have poor chances to disturb its folding, especially because it is stiffened by the four disulfide bridges. We then assume that both forms may present a rather similar activity.

Native mapacalcine has been described as a dimer linked by at least one disulfide bridge [7]. We also detected it as a dimer by dissociating mass spectrometry like MALDI-TOF before DTT treatment and as a monomer after DTT treatment, confirming that the dimer was linked by at least one disulfide bridge (not shown). The recombinant mapacalcine was detected as a monomer by the same technique. Gentle technics like LSA an SEC demonstrated a molecular weight consistent with a monomer. Taken together, these observations suggest that no dimeric form of mapacalcine even linked by non-covalent interactions was produced. The presence of a 300 Da molecular entity bound to a free cysteine is consistent with the idea that this unpaired cysteine may be modified by a glutathione, a current feature in protein production in *P. pastoris* when methanol is used as inducer [28,29]. This may explain why only the monomeric form of the protein is obtained. All the other cysteines being engaged in intramolecular bridges, the ninth cysteine (theoretically unpaired) of two different monomers cannot react together to form a dimer. When using an enzymatic deglutathionylation to selectively remove such an entity while preserving the disulfide bridges, we failed to clearly evidence the presence of a glutathione (data not shown). This may indicate that the concerned cysteine remains inaccessible to enzymes. Further productions, avoiding the presence of inducers such as methanol, are actually in progress. Nevertheless, the recombinant monomeric mapacalcine production described in this work provides a convenient tool for further applications such as receptor identification, immuno-histology, anti-ischemic applications. The fact that it has similar biological properties as the natural (dimeric) mapacalcine suggests (i) that the tertiary structure of the monomer of recombinant mapacalcine is probably similar to the one of the native protein, the formation of the dimer being prevented by the blocking of the cysteine by a glutathione. (ii) That it retains the essential structural domains required for its activity. These properties evidence its usefulness to conduct site directed mutagenesis and therefore to identify the minimum pattern responsible for the biological activity of the protein.

Acknowledgements

The authors are grateful to Dr. Marie-France Giraud (IBGC, UMR 5095, Univ. Bordeaux) for light scattering facilities.

This work was funded by Université de Bordeaux, AAP2014.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.10.003.

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