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

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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.

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## 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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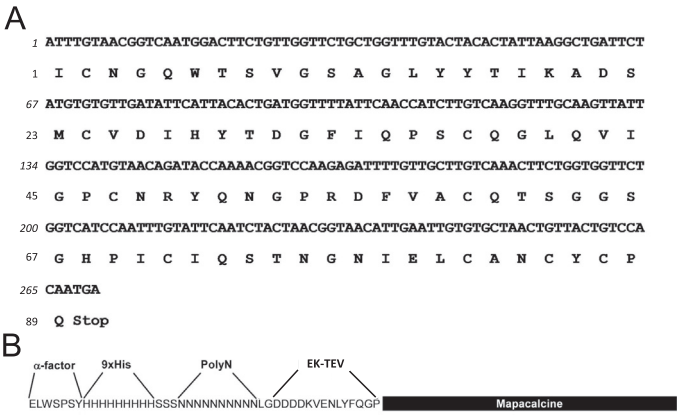


**Fig. 1.** Synthetic gene construction of mapacalcine from its aminoacid sequence. A: Mapacalcine monomer (89 aminoacids) and its deduced synthetic gene sequence (270 nucleotides). B: Map of the expressed 9HIS-mapacalcine cassette inserted in *Pichia pastoris* genome.

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, $10\times250$ 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.

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