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In vitro cell free synthesis of human manganese superoxide dismutase with the RTS 500 system

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Abstract

Reduced activity of manganese superoxide dismutase (MnSOD) is the basis of several pathologic features and complications occurring in the course of infectious mononucleosis. In order to supply future research with easily accessible enzyme, an in vitro protocol was developed based on the RTS 500 system and an overexpression vector. Translation of MnSOD monomers could be detected by SDS–PAGE, and assembly of the active homotetramer by native PAGE. Enzyme activity was successfully shown by in gel activity tests and enzyme assays. With 15 μg of DNA, 2.45 μkat were generated. The purification of MnSOD was performed by chromatography applying the His-tag technology. In SDS–PAGE of the eluate, a band showed up at M_r 25 000.

Keywords: Epstein–Barr virus; Enzymes; Manganese superoxide dismutase

1. Introduction

Superoxide (O_2^-) is a cytotoxic reactive oxygen species (ROS) damaging proteins that contain iron–sulphur centres. It is generated in the course of the breakdown of cellular purines by xanthine oxidase and as a by-product in the mitochondrial electron transport chain [1,2]. In mammals, three different enzymes have evolved for the detoxification of ROS: extracellular Cu/Zn superoxide dismutase, cytosolic Cu/Zn superoxide dismutase and intermitochondrial manganese superoxide dismutase (MnSOD), which has also been found in the cytosol and in serum [3,4].

Human MnSOD is encoded by five exons on chromosome six. Two different mRNAs have been identified containing an open reading frame of 651 basepairs and differing only in the length of the 3′-untranslated region [5]. The translated protein is transported into the mitochondria via an aminoterminal targeting sequence. The active enzyme is a 96-kDa homotetramer with one Mn^{3+} per 24-kDa monomer. It is the major antioxidant in mitochondria catalyzing the reaction $2\ H^+ + 2\ O_2^- \leftrightarrow H_2O_2 + O_2$.

MnSOD is essential for cellular survival. A deletion mutation of the related gene in mice results in death within 5–21 days of birth [6,7]. Pathological features exhibited by these mice include myocardial injury, neurodegeneration, lipid peroxidation, anaemia, and severe mitochondrial damage. This is not the case for other members of the dismutase family [8,9]. Heterozygous knockout mice survive, but show oxidative damage and alterations in mitochondrial function [10]. Altered MnSOD levels have

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been demonstrated in several pathologic processes: MnSOD is inactivated by tyrosine nitration during human chronic allograft rejection [11]. Apoptosis in neuronal cells due to an excess of iron, amyloid β-peptide, or nitric oxide donors can be prevented by elevated MnSOD levels [12]. Furthermore, in influenza virus infected mice treated with recombinant MnSOD pulmonary toxicity was alleviated [13]. Ritter and co-workers [14] have shown that autoantibodies against MnSOD are produced during acute Epstein–Barr virus (EBV) infection. MnSOD activity was found to be reduced allowing cell and tissue damage which can explain the major complications in acute EBV infections.

Further research on the role of MnSOD in pathologic processes, especially in acute EBV infections, requires larger amounts of the enzyme. However, human MnSOD is not commercially available. It can be isolated from human liver by a multistep chromatographic procedure or it can be overexpressed in *E. coli* [15], both of which are very time consuming. Furthermore, human liver tissue is difficult to obtain. In this study we present a protocol for convenient in vitro synthesis of human MnSOD.

2. Experimental

2.1. Construction of MnSOD-expression vectors

Total RNA was isolated from 10^5 cells of the Burkitt lymphoma cell line Raji with the RNeasy kit (Qiagen, Hilden, Germany) and reversely transcribed with the First Strand cDNA Synthesis kit (Amersham Biosciences, Little Chalfont, UK). The open reading frame (ORF) for human MnSOD was amplified from the cDNA (2 min at 94 °C; 35 cycles of: 30 s at 94 °C, 30 s at 54 °C, 45 s at 72 °C; 7 min at 72 °C) using the primers MnSOD-Nco (GCA CTA GCA CCA TGG TGA GCC GGG CAG TGT G), MnSOD-Sma-OS (AAC GAT CGT CCC GGG CTT TTT GCA AGC CAT GTA TC) and MnSOD-Sma-MS (CAT AAC GAT CCC GGG TTA CTT TTT GCA AGC CAT GTA TC). The amplicon for the primer combination MnSOD-Nco/MnSOD-Sma-MS contained the entire MnSOD-ORF with the consensus sequence for NcoI at the 5′-end and for SmaI at the 3′-end for ligation into the vector pIVEX2.4b. The amplicon for the primer combination MnSOD-Nco/MnSOD-Sma-OS contained the MnSOD-ORF lacking the stop codon with the consensus sequence for NcoI at the 5′-end and for SmaI at the 3′-end for ligation into the vector pIVEX2.3. Both vectors were restricted with NcoI and XmaI and ligated to the related PCR-product resulting in the vectors pIVEX2.4b-MnSOD and pIVEX2.3-MnSOD.

2.2. In vitro synthesis of MnSOD with the RTS 500 system (Roche Applied Sciences, Mannheim, Germany)

The reaction solution was prepared by mixing 525 μl *Escherichia coli* lysate, 225 μl reaction mix, 270 μl amino acid mix, 30 μl methionine solution, and 15 μl DNA solution (1 μg/μl pIVEX2.4b-MnSOD or pIVEX2.3-MnSOD). The feeding solution was prepared by mixing 2.65 ml amino acid mix, 0.3 ml methionine solution, and 8 ml feeding mix (all components from Roche Applied Sciences, Mannheim, Germany). Reaction solution and feeding solution were filled into the appropriate compartments of the reaction device separated by a semipermeable membrane. The reaction device was placed into the RTS 500 instrument and was incubated for 24 h at 30 °C and 120 rpm.

Batch reactions were performed by filling 50 μl of the reaction solution into standard reaction tubes and incubating them for 1 h at 30 °C.

2.3. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

For SDS–PAGE, samples were separated according to the method of Laemmli [16] with the Mighty Small system (Hoefer Scientific Instruments, San Francisco, USA) for 1 h at 16 mA. For native PAGE SDS in all Laemmli buffers was replaced by water. Unspecific protein detection was done by Coomassie blue staining. Proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF)-membranes (Millipore, Schwalbach, Germany) at 25 V for 16 h. Blot membranes were incubated with serum of anti-MnSOD positive patients (1:50) and peroxidase-conjugated anti-human IgM antibodies (1:1000, Dako, Hamburg, Germany) as second anti-

body. The blots were developed with diaminobenzidine hydrochloride as peroxidase substrate.

2.4. Superoxide dismutase activity assays

For quantification of superoxide dismutase activity in aqueous solutions 3 ml assay mix (0.01 m*M* cytochrome *c*, 0.05 m*M* Na-xanthine, 0.08 m*M* EDTA, 6.25 m*M* K-phosphate buffer pH 7.8) were mixed with 6 μl sample. The reaction was started by adding 0.02 U xanthine oxidase. Absorbance at 550 nm and room temperature was monitored for 2 min and ΔA_{550}/min was calculated. Reduction of ΔA_{550}/min by 50% in samples compared to buffer-controls was defined as 1 enzyme U MnSOD corresponding to a catalytic activity of 16.67 nkat [17].

For association of catalytic activity with a protein of defined molecular mass a native PAGE was performed. The gel was incubated in 1 ml NBT-buffer A (0.025% nitroblue tetrazoliumchloride, 0.01% riboflavin) per 50 cm^2 of gel for 20 min at room temperature in the dark. The incubation was repeated with NBT-buffer B (1% TEMED) under the same conditions. The sample was incubated in light until a blue background staining was visible. Regions of superoxide dismutase activity could be identified as unstained areas.

2.5. Purification of MnSOD

Purification of in vitro synthesized human MnSOD was done by chromatography applying the His-tag technology. The translation samples were treated with the Talon Metal Affinity Resins kit (Becton Dickinson Biosciences, Palo Alto, USA) and the Ni-NTA Spin kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Further purification was done with the HiTrap Chelating HP kit (Amersham Biosciences, Little Chalfont, UK). First 100 μl of the MnSOD gained by the translation assay were mixed with 900 μl binding buffer (0.02 *M* sodium phosphate, 0.5 *M* NaCl, 8 *M* urea, pH 7.4) and then incubated on ice for 30 min. The chromatography column was loaded with Ni (10 ml H_2O, 0.5 ml 0.1 *M* $NiSO_4$, 10 ml H_2O, 1 ml/min for each step) and calibrated with 10 ml binding buffer. The column was loaded with 1 ml of the translation assay product and washed with 7 ml binding buffer. Finally, the protein was eluted in five consecutive steps each using 1 ml elution buffer with increasing imidazole concentrations (0.02 *M* sodium phosphate, 0.5 *M* NaCl, 8 *M* urea, pH 7.4; 0.125, 0.15, 0.375, 0.5, and 0.5 *M* imidazole, respectively). The flow was collected in 1-ml aliquots. Then 15 μl of each aliquot were analyzed by immunoblotting.

3. Results and discussion

3.1. Construction of MnSOD-expression vectors

For construction of MnSOD-expression vectors the open reading frame (ORF) of human MnSOD was amplified from cDNA of Burkitt lymphoma cells resulting in a 651-bp product with the primer pair MnSOD-Nco/MnSOD-Sma-MS and a stop codon-less 648-bp product with the primer pair MnSOD-Nco/MnSOD-Sma-OS. The amplicons were ligated to the expression vectors pIVEX2.4b and pIVEX2.3, respectively, resulting in pIVEX2.4b-MnSOD and pIVEX2.3-MnSOD (Fig. 1). For convenient purification of the gene product both vectors contained a His-tag. pIVEX2.4-MnSOD codes for an MnSOD fusion protein with a His-tag at the amino terminus. The stop codon is supplied by the ORF of MnSOD. pIVEX2.3-MnSOD codes for a fusion protein with a His-tag at the carboxy terminus. In order to allow the generation of the fusion protein, the MnSOD stop codon was abolished during the amplification- and cloning procedure. Translation is terminated by the vector-encoded stop codon downstream of the His-tag.

3.2. In vitro synthesis of human MnSOD

For synthesis of MnSOD the novel in vitro translation system RTS 500 was used. The vectors pIVEX2.3-MnSOD and pIVEX2.4b-MnSOD were tested for translation efficiency in a 50-μl batch reaction. Plasmid DNA was incubated for 1 h with the reaction solution containing *E. coli* extracts. The samples were analyzed by immunoblotting using patient sera positive for anti-MnSOD antibodies. The batch reaction proved a higher translation efficiency for pIVEX2.4b-MnSOD (data not shown). This vector was used for a large scale in vitro translation

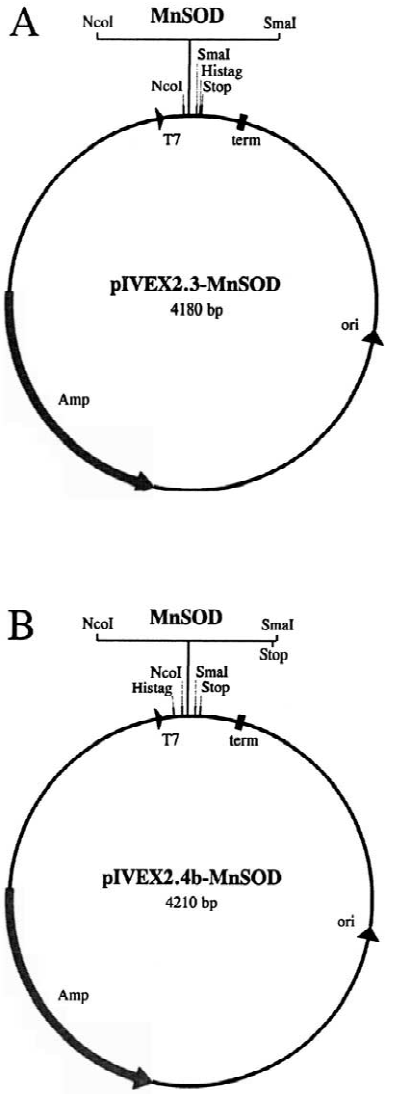

Fig. 1. Plasmid maps of pIVEX2.3-MnSOD (A) and pIVEX-2.4b-MnSOD (B). Amp, β-lactamase gene; ori, origin of replication; MnSOD, ORF of human manganese superoxide dismutase; T7, T7-promoter; term, termination sequence from T7; Stop, stop codon.

reaction. The reaction device was filled with 1 ml reaction solution and 8 ml feeding solution which are separated by a semipermeable membrane. After incubation for 24 h the solution from the reaction compartment was removed and further analyzed. Immunoblotting on an SDS–polyacrylamide gel revealed a prominent band at M_r 25 000 (MnSOD+ His-tag) which corresponds to a Coomassie-stained band. The control reaction without DNA showed no band of this size either in the Coomassie staining or in the immunoblot (Fig. 2). Immunoblotting of a native polyacrylamide gel identified a protein of M_r 100 000 representing the MnSOD/His-tag fusion protein homotetramer and aggregates of higher molecular mass. The control reaction did not show a signal at that size (Fig. 3).

The results prove the successful in vitro synthesis of MnSOD using the RTS 500 system. The absence of signals in the control sample excludes *E. coli* MnSOD as the causative agent for the signal in the overexpression sample. Furthermore, active human MnSOD forms a homotetramer, whereas the active *E. coli* enzyme is a homodimer [18,19]. Thus, the detection of a band of the size of the homotetramer in native PAGE is the final proof of uncontaminated in vitro synthesis of human MnSOD.

3.3. Activity assays with recombinant human MnSOD

Human MnSOD activity assays were performed for the crude translation reaction and in gel with

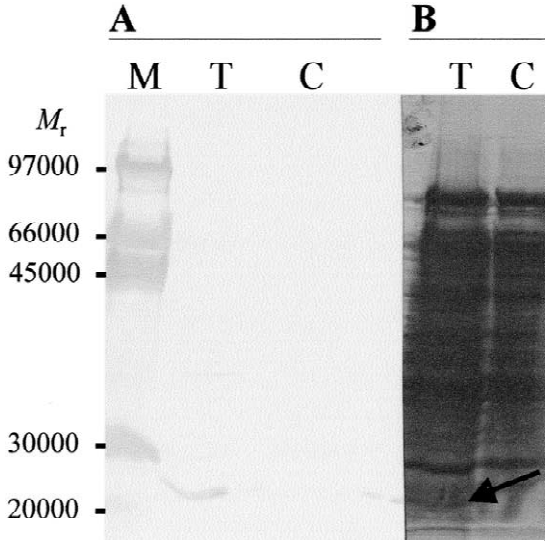

Fig. 2. Immunoblot (A) and Coomassie staining (B) of SDS–PAGE of MnSOD in vitro translation assays using anti-MnSOD positive patient sera. C, control translation assay; M, size marker; T, translation assay with pIVEX2.4b-MnSOD.

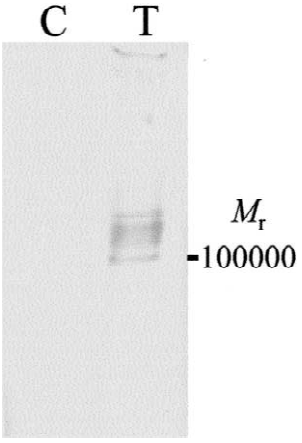

Fig. 3. Immunoblot of native PAGE of MnSOD in vitro translation assays using anti-MnSOD positive patient sera. C, control translation assay; T, translation assay with pIVEX2.4b-MnSOD.

separated proteins. The in-gel assay with NBT was performed following native polyacrylamide gel electrophoresis. An unstained region could be observed proving MnSOD activity. The region correlated with the signal of M_r 100 000 from the immunoblot of the native gel (Fig. 4). No MnSOD activity was observed in the control reaction. MnSOD activity in the crude translation reaction was assayed with a xanthine/xanthine oxidase system. Absorbance at 550 nm was reduced by 0.0075 per minute with the MnSOD translation sample compared to the control sample. This represents an MnSOD activity of 14.7 nkat per 6 μl sample according to McCord and Fridovich [17]. Thus, the total reaction revealed 2.45 μkat human MnSOD activity.

The results of the activity assay show that the translation assay indeed contains human MnSOD activity. This activity is associated with the MnSOD homotetramer. Isolation of MnSOD from human liver by a multistep chromatographic procedure can result in 150 μkat per 100 g of tissue [20]. This yield exceeds the yield of the in vitro synthesis by factor 60. However, as human liver is difficult to obtain and the isolation procedure is very time consuming and laborious, the successful in vitro synthesis is very promising for future research.

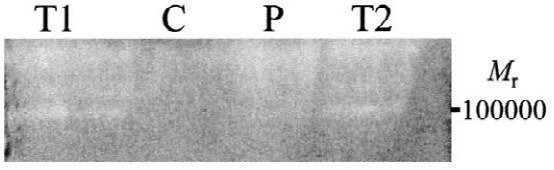

Fig. 4. MnSOD activity assay of a native PAGE of MnSOD in vitro translation assays using an NBT-assay. C, control translation assay; P, positive control; T1, T2, different translation assays with pIVEX2.4b-MnSOD.

3.4. Purification of recombinant human MnSOD

Purification of the MnSOD gained by translation was tried by chromatography applying the His-tag technology. Native MnSOD translation samples and denatured translation samples were treated with the Talon Metal Affinity Resins kit and the Ni-NTA Spin kit. No MnSOD specific band could be detected by immunoblotting. Translation samples denatured with 8 *M* urea were purified with the HiTrap Chelating HP kit. Proteins were eluted in five fractions with increasing imidazole concentrations. Coomassie staining of SDS–PAGE of each fraction revealed protein signals only in the 125 m*M* imidazole fraction and the 150 m*M* imidazole fraction. A band at M_r 25 000 could be identified by immunoblotting with MnSOD-specific sera only in the 375 m*M* imidazole fraction (Fig. 5).

This shows that human MnSOD was successfully purified from the crude extract into one fraction without any protein contamination detectable by

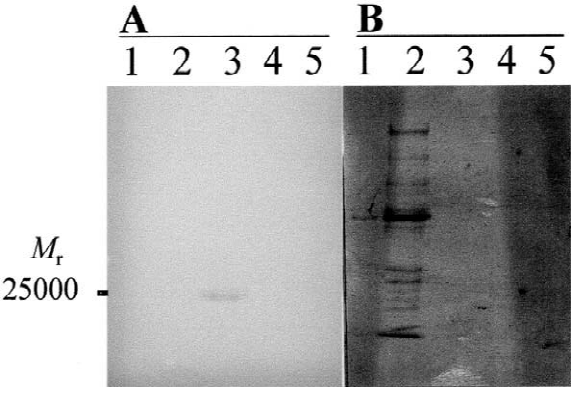

Fig. 5. Immunoblot (A) and Coomassie staining (B) of MnSOD in vitro translation assays purified with the HiTrap Chelating HP kit. 1–5: aliquots of protein fractions eluted with increasing imidazole concentrations (1: 125 m*M*, 2: 150 m*M*, 3: 375 m*M*, 4: 500 m*M*, 5: 500 m*M*).

Coomassie staining. Due to the low MnSOD concentration the enzyme was not detectable by Coomassie staining. For this reason protein contamination can be finally ruled out only by using a more sensitive method of silver staining. The method described is a novel way for in vitro synthesis and purification of MnSOD avoiding human liver as a source of the enzyme and reducing the purification time significantly.

Purification of in vitro translated MnSOD with the His-tag technology proved to be difficult. Purified protein could be detected only with the HiTrap Chelating HP kit. Another method is the well established multistep chromatographic protocol of Matsuda and co-workers [21]. The small volume of the sample requires chromatography columns of reduced size, thus speeding up the procedure. Alternatively, we suggest that the homotetrameric human MnSOD can easily be separated from the dimeric *E. coli* MnSOD and human MnSOD aggregates of higher molecular mass by gel filtration due to its size. However, this method may be inefficient for removal of contaminating proteins with a molecular mass close to that of human MnSOD.

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