

Cloning, Expression and Purification of Human S-Adenosylmethionine Decarboxylase Gene α Subunit

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Abstract

S-adenosylmethionine decarboxylase (SAMDC) is an essential enzyme for the synthesis of spermidine and spermine in the biosynthetic pathway of polyamines. The total RNA was extracted from colon cancer tissue and amplified by reverse-transcription PCR with two primers, which span the coding region of SAMDC α subunit. Clone vector pMD18-T-SAMDC- α was successfully constructed by using T-A clone technique. pMD18-T-SAMDC- α and pTriEx-4 were digested by *Nco*I and *Xho*I double enzymes. The purified SAMDC- α fragment was subcloned into the expression vector pTriEx-4 to construct the prokaryotic expression plasmid pTriEx-4-SAMDC- α . The recombinant plasmid pTriEx-4-SAMDC- α was transformed into competence *E.coli* JM109 (DE3). The bacterium was induced by IPTG and its lysates were loaded directly onto SDS-PAGE. An approximately 32 kDa exogenous protein was observed on the SDS-PAGE. The protein was verified by Western blot with anti His-Tag monoclonal antibody. The fusion protein including 6 \times His-Tag was purified by Ni-NTA chromatographic column. Then, the purified protein can be applied for further research of the immunity of SAMDC.

Key Words: S-adenosylmethionine decarboxylase, prokaryotic expression

Introduction

The human S-adenosylmethionine decarboxylase (SAMDC) is an essential enzyme for the synthesis of spermidine and spermine (19), consisting of a dimer of two non-identical subunits $\alpha_1\beta_2$. SAMDC catalyzes the formation of decarboxylated S-adenosylmethionine, which acts as the aminopropyl donor for the biosynthesis of the polyamines, spermidine, and spermine. This decarboxylation reaction is one of the key and rate limiting steps in polyamine biosynthesis (3). Spermidine and spermine are essential regulating factors for cell growth and play an important role in carcinogenesis and neoplastic cell proliferation. Enhanced proliferation in colon cancer cells is associated with increased SAMDC activity and rapid conversion of putrescine to spermidine and spermine (6, 9). SAM486A as an inhibitor of SAMDC was applied to study the

suppression effect on cancer cell growth (5, 11). To study the immunity of α subunit, we constructed a prokaryotic expression vector of SAMDC α subunit which includes 267-amino acids. The 6 \times His-tag fusion protein was induced by isopropyl- β -thiogalactoside (IPTG) efficiently in *Escherichia coli*. The 6 \times His-tag enabled us to purify the fusion protein by Ni-NTA chromatographic column with high purity. The successful expression of SAMDC α subunit fusion protein facilitated the further study of SAMDC.

Materials and Methods

Strains and Reagents

E.coli strain of JM109 (DE3) and DH5 α were conserved in our laboratory. The prokaryotic expression vector pTriEx-4 vector, was kindly provided by Prof.

Guangshui Jiang (Stomatological School of Shandong University). RT-PCR kit, T-A clone kit, DNA marker, restrictive enzymes (*Nco*I and *Xho*I), pMD18-T vector, T4 DNA ligase and Minibest plasmid purification kit were purchased from TaKaRa (Dalian, Liaoning, China). Trizol (RNA extract reagent), agarose and IPTG were purchased from Shanghai Sangon Bioengineering Co. Ltd (Shanghai, China). The Qiaquick gel extraction kit was purchased from Qiagen (Valencia, CA, USA). Anti His-Tag monoclonal antibody was purchased from Novagen (Madison, WI, USA). His Trap™ kit (for purification of histidine tagged proteins) was purchased from Amersham (Pittsburgh, PA, USA).

Extraction of Total RNA

The total cellular RNA was extracted from colorectal carcinoma. The method of RNA extraction was similar to the Trizol RNA extraction protocol.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The sequence of SAMDC α subunit primers was designed as follows, up-stream primer: 5'-CCCATGGCAAGTAGCATGTTTGTCTCCA-3' (*Nco*I site in underline); down-stream primer: 5'-TTCTCGAGACTCTGCTGTTGTTGCTGC-3' (*Xho*I site in underline). Reverse transcription was performed at 55°C for 30 min, 99°C for 5 min, and then 5°C for 5 min. A portion (2 μ l) of the cDNA product was amplified by PCR with Taq DNA polymerase. PCR reactions (35 cycles) were 94°C for 40 sec, 57°C for 40 sec, 72°C for 1 min 30 sec with initial inactivation of enzyme at 94°C for 5 min and another 7 min for elongation at 72°C after the cycles were finished. The amplification products were analyzed by electrophoresis on 1% agarose gel.

Purification of PCR Product and T-A Cloning

After the PCR product was gel purified, the ligation was performed between the target gene and the pMD18-T vector overnight at 16°C, then transformed into *E. coli* DH5 α competent cells and selected by selective culture medium containing ampicillin. For screening purposes, the positive colony with the target gene in the plasmid was identified by double restriction digestion (*Nco*I and *Xho*I), followed by agarose gel analysis, and conformed further by DNA sequencing (Shanghai Sangon Bioengineering Co. Ltd.).

Construction of pTriEx-4-SAMDC- α

The pMD18-T-SAMDC- α and pTriEx-4 were

digested by *Nco*I and *Xho*I. The inserted fragment of pMD18-T-SAMDC- α was collected from electrophoretic gel, and was ligated with the linearized pTriEx-4 by T4 ligase at 16°C overnight. The recombinant was transformed into *E. coli* JM109 (DE3) competent cells and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping.

Expression of SAMDC α Subunit Fusion Protein

The ampicillin-resistant colony of *E. coli* JM109 (DE3) cells with recombinant plasmid were cultured at 37°C in LB cultural medium containing 100 mg/l ampicillin, and induced by 1 mM IPTG. The cultured cells were harvested at 1, 2, 3, 4, 5 h, respectively after culture. The optimum time of maximum expression of proteins was analyzed through SDS-PAGE. The expressed SAMDC α subunit fusion protein was tested through Western blot with mouse anti His-Tag monoclonal antibody.

Purification of SAMDC α Subunit Fusion Protein

The histidine-tagged fusion protein can be purified by affinity chromatography. The *E. coli* JM109 (DE3) cells with recombinant plasmid were cultured at 37°C in 100 ml LB cultural medium containing 100 mg/l ampicillin, and induced by 1 mM IPTG at the optimum time of maximum expression, and then the cells were harvested by centrifugation. The cells were resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8.0, 8 mol urea), and the ratio of buffer/cells was 5 ml/g. Cell debris was removed by centrifugation at 4°C, 17,000 \times g, 15 min. The pellet was discarded. Then the supernatant was loaded into the chelating column which had been loaded 0.5 ml NiSO₄, and the flow-through fraction was collected the column was washed with 10 ml binding buffer to collect the wash fraction. At last, the column was eluted with a step gradient of imidazole concentrations of 20 mM, 40 mM, 60 mM, 100 mM, 300 mM, 500 mM in 5 ml elution buffer, respectively and then collected in 1 ml fraction to avoid dilution.

Results

RT-PCR and Cloning

RT-PCR was done with total RNA template extracted from human colon cancer. The designed primers include encoding sequence of SAMDC α subunit. Electrophoresis of RT-PCR products confirmed the length of RT-PCR fragment (801 bp) (Fig. 1).

The purified SAMDC α subunit cDNA was ligated to pMD18-T vector by T-A complimentary pairing. SAMDC α subunit cDNA was inserted into

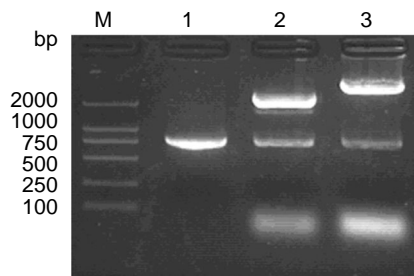


Fig. 1. Electrophoresis figure of pTriEx-4-SAMDC- α construction. Lane M: DNA marker/DL2000; Lane 1: RT-PCR product of SAMDC- α ; Lane 2: pMD18-T-SAMDC- α digested by *Nco*I and *Xho*I; Lane 3: pTriEx-4-SAMDC- α digested by *Nco*I and *Xho*I.

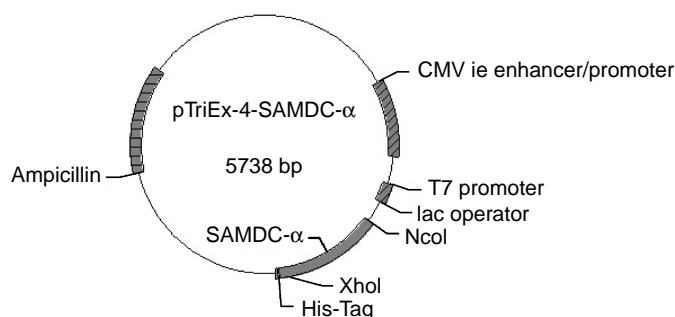


Fig. 2. The pTriEx-4-SAMDC- α expression vector including T7 promoter, lac operator and His-Tag. SAMDC α subunit was inserted into pTriEx-4 at restrictive enzymes *Nco*I and *Xho*I sites.

pTriEx-4 at *Nco*I and *Xho*I (Fig. 2).

Sequence Analysis

Sequence of inserted DNA was analyzed with automatic sequence analyzer and showed 99% affinity in comparison with DNA sequence published on line (gi:5209326).

SDS-PAGE and Western Blotting

Inserted SAMDC α subunit gene was expressed significantly in the prokaryotic expression system. The optimum induction period is 4 h after administration of IPTG (Fig. 3). A specific strip at 32 kDa was demonstrated by Western blot analysis using mouse anti His-Tag monoclonal antibody (Fig. 4). So the recombinant protein was the histidine-tagged fusion protein.

Purification

The optimum condition for elution was the elution buffer of which the imidazole concentration

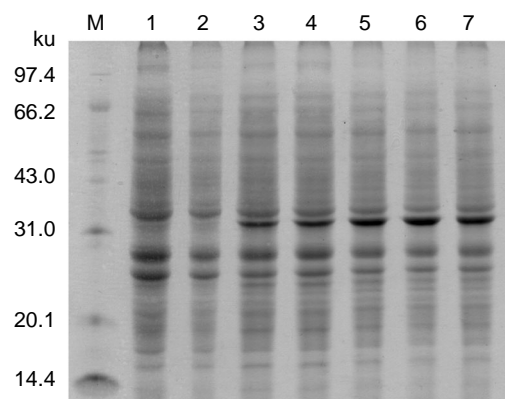


Fig. 3. SDS-PAGE figure of pTriEx-4-SAMDC- α induced in JM109 (DE3). Lane M: low molecular mass protein marker; Lane 1: JM109 (DE3) without pTriEx-4-SAMDC- α , as control; Lane 2: pTriEx-4-SAMDC- α expression result before induction; Lane 3~7: pTriEx-4-SAMDC- α expression results with IPTG induced 1 h, 2 h, 3 h, 4 h, 5 h, respectively.

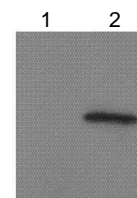


Fig. 4. Western blot analysis of the expression product of the pTriEx-4-SAMDC- α with mouse anti His-Tag monoclonal antibody. Lane 1: mock; Lane 2: fusion protein.

was 300 mM. The maximum protein was in the second tube of eluting fraction. The purified proteins were performed on SDS-PAGE and obtained approximately 32 kDa strip which was consistent with the size of SAMDC α subunit (Fig. 5). Western blot with specific antiserum showed that the protein was exactly SAMDC- α (Fig. 6).

Discussion

The polyamines putrescine, spermidine and spermine are organic cations shown to participate in a bewildering number of cellular reactions (7). Their positive charge enables polyamines to interact electrostatically with polyanionic macromolecules within the cell, such as DNA and RNA (18). Many studies have shown that polyamines are essential for cell survival and proliferation (9, 14), with high concentrations being found in rapidly growing cells and tissues (12). Cancer cells always have higher intracellular polyamine content than the equivalent normal tissue (1, 2, 17). A large body of data indicates

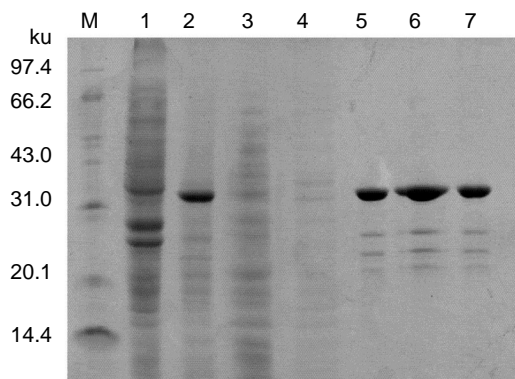


Fig. 5. SDS-PAGE figure of purified SAMDC- α protein. Lane M: low molecular mass protein marker; Lane 1: JM109 (DE3) without pTriEx-4-SAMDC- α , as control; Lane 2: cell lysate of JM109 (DE3) with pTriEx-4-SAMDC- α induced for 4 h; Lane 3: flow-through fraction; Lane 4: wash fraction; Lane 5~7: purified SAMDC- α protein eluted by 300 mM imidazole 1 ml, respectively.

that polyamine pathway can be a molecular target for therapeutic intervention in several types of cancers (14).

There are two critical enzymes controlling the polyamine metabolic pathway, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC). ODC is the first rate-limiting enzyme in the biosynthesis of polyamines and has been the major target for investigation (15). Our previous study also showed that the ODC gene expression is higher in colorectal carcinoma than that in normal coloractal tissues (4). Difluoromethylornithine (DFMO), a synthetic and irreversible inhibitor of ODC, which has been evaluated for its anticancer activity in advanced disease and is currently being investigated as a chemopreventive agent in premalignant disorders (13). On the other hand, SAMDC, the second rate-limiting enzyme, has been implicated to play a predominant role in tumor growth by promoting the formation of the more distal polyamines spermidine and spermine (8). Inhibitors of SAMDC were found to suppress tumor formation in experimental models of bladder, breast, colon, and skin carcinogenesis (5, 10, 11).

Human SAMDC is synthesized as a 38.3 kDa proenzyme which subsequently undergoes processing at amino acid 68 to yield two fragments 32 kDa (α) and 6 kDa (β). The mature human SAMDC consists of a dimer of these two non-identical subunits $\alpha_2\beta_2$ (16). To study the immunity of α subunit, we only constructed α subunit expression vector. This could also avoid the proenzyme processing, so we could predigest the purification of fusion protein. We designed two primers from human SAMDC mRNA to

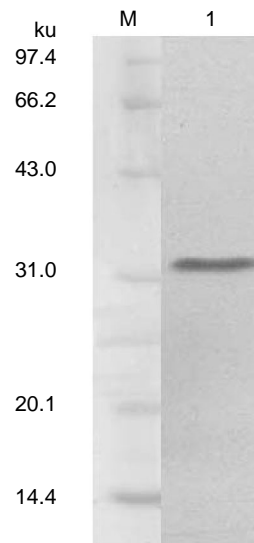


Fig. 6. Western blot analysis of purified protein with specific antiserum of SAMDC. Lane M: low molecular mass protein marker; Lane 1: purified protein.

amplify the whole encoding sequence from 522 bp to 1322 bp of SAMDC α subunit. The 801 bp sequence encoding a protein is identical to the 267-amino acid sequence derived from human SAMDC. The restriction fragment mapping of the recombinant, pTriEx-4-SAMDC- α , indicated that the inserted fragment was about 800 bp, which was consistent with the encoding sequence of SAMDC α subunit. The expressed fusion protein is about 32 kDa, and which is conformed to the known SAMDC α subunit protein. The successful construction and correct expression of recombinant SAMDC α subunit prokaryotic expression vector provide a potent tool for related further study.

Acknowledgments

We would like to thank Dr. Guangshui Jiang for donation of pTriEx-4 plasmid and Dr. Chuanyong Liu for revising the manuscript. This work is supported by Foundation of Medicine and Health of Shandong Province (2001CA1CAA3).

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