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## CLONING, EXPRESSION AND PURIFICATION OF HUMAN TRUNCATED SIRT1

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**Abstract-** Sirtuins belong to an evolutionarily conserved class of proteins that regulate a variety of cellular functions such as genome maintenance, longevity, and metabolism. The conserved part of amino acids residues 244-498 of full length human SIRT1 have the functional activity. To construct a functional form of SIRT1 by DNA recombinant technology, we have expressed and purified truncated protein of 420 (193 to 611) amino acids of MW 47 kDa in prokaryotic system, *E. coli* BL21 (DE3) strain. This functional protein has a potential to be used as a diagnostic tool and for the development of new therapeutic agent for age related disease.

**Key words-** Truncated SIRT1; Protein expression; Mass Spectroscopy; Western blot.

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### Introduction

Sirtuin or silence information regulator comprises an universal conserved family of NAD<sup>+</sup> (Nicotinamide adenine dinucleotide) dependent deacetylases conserved from bacteria to human and play a role in a wide variety of important biological processes including development, heterochromatin formation, transcriptional silencing, DNA recombination and repair, genomic stability, apoptosis, axonal protection, fat metabolism, metabolic regulation and longevity [1,2]. This enzyme was first isolated from yeast, and later it was found to be present in mammals including humans. There are seven human sirtuins (1-7) that display diversity in cellular localization and function. They contain a conserved catalytic core domain comprising of approximately 275 amino acids. SIRT1 is most studied among the 7 sirtuin family member, is highly expressed in several adult tissue such as brain, heart and skeletal muscle [3]. Calorie restriction, the only known mechanism which has been reported to delay ageing is mediated by sirtuin gene family which were shown to have anti ageing function in yeast [4], *Caenorhabditis* [5], *Drosophila* [6] and mammals including rodents [7], and perhaps primates [8, 9]. SIRT1 is localized in the nucleus [10, 11] and cytoplasm [12]. It interacts and deacetylates many proteins like p53 [10, 11], the forkhead transcription factor

FOXO3A [13], BCL6 [14], TAF68 [15], HES1 [16] and CTIP2 [17]. It is an important mediator of longevity through induction of cell cycle arrest and resistance to oxidative stress by deacetylating FOXO3. Sirtuins have emerged as a therapeutic target to treat age related diseases. SIRT1 controls insulin secretion from pancreatic beta cells via UCP2, FOXO1 and NAD metabolism and can help on the development of new therapeutic tool for insulin resistance and type2 diabetes [18]. It promotes fatty acid mobilization in white adipose tissues by deacetylating and repressing PPAR $\gamma$  (Peroxisome proliferator activated receptor  $\gamma$ ) [19]. In skeletal muscles, it results in increased aerobic capacity [20, 21] while in liver it increases gluconeogenesis [22] by deacetylating and activating PGC1 $\alpha$  (PPAR1 $\gamma$  coactivator 1 $\alpha$ ). Three different mRNA sequence of human SIRT1 are known from Gene bank NM\_012238 (4110 bp), NM\_001142498.1 (3604 bp) and BC012499.1 (3691 bp) respectively, with their respective frames encoding 747, 452 and 555 amino acids residues. The preliminary crystal structure of truncated SIRT1 from 140-747 amino acid residues is also reported [23]. In the present study, we report the cloning, expression and purification of truncated human sirtuin constructed from 193- 611 amino acid residues.

## Materials and methods

### Bio-informatics Analysis

mRNA sequence of human SIRT1 gene was obtained from NCBI Gen Bank ([www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) under accession No. NM\_012238. NCBI ORF finder ([www.ncbi.nlm.nih.gov/projects/gorf/](http://www.ncbi.nlm.nih.gov/projects/gorf/)) was used to find out the ORF and respective amino acid sequence. The deduced amino acids sequence was analyzed by ExPasy MW/pI ([web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)) to calculate the molecular weight and pI of the protein. Homologues were explored by BLAST programme ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) in NCBI Gen Bank database and multiple sequence alignment was performed using Clustal W programme. ([www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)).

### Cloning

#### PCR

Truncated SIRT1 gene was amplified by using Pfu Taq Polymerase (Phusion). The forward and reverse primers used for the amplification were CGGGATCATGATTGGCACAGATCC and CCGCTCGAGCTATTCATTTTCTCCCC respectively having BamH1 and Xho1 sites.

#### Cloning into pGEMT easy vector

The amplified product was purified using PCR cleanup kit (Promega) and adenylated by Taq polymerase followed by cloning into pGEMT easy vector (Promega). The ligation mixture was transformed into *E.coli* DH5 $\alpha$  competent cells and positive clones were identified by blue white screening. The sequence of positive clones was confirmed by PCR-based sequencer.

### Sub-cloning into expression vector

Plasmid was extracted from the positive clones by using Plasmid Purification Kit (Promega). Directional cloning was performed by using BamH1 and Xho1 as a restriction sites to clone truncated SIRT1 gene into pET28a<sup>+</sup> vector using T4DNA ligase. Ligation mixture was then transformed into expression host *E.coli* BL21 (DE3) competent cells.

### Expression

*E.coli* BL21 cells possessing recombinant plasmid were allowed to grow in 100 ml of LB media containing Kanamycin (50 mg/L) at 37°C until absorbance at 600 nm reached 0.6 and then induced with 1 mM IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside). The cells were harvested after 5 hrs of induction and weighed. The induced and uninduced cultures were analysed by SDS- PAGE to ensure the recombinant protein expression.

### Protein purification and on column refolding

Inclusion bodies obtained after sonication of the 0.3 grams of harvested cells in 1.2 ml of 50 mM Tris-HCl were re-suspended in Buffer A (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0 and 8 M urea), centrifuged and the supernatant was loaded on 1 ml of Ni-NTA resin (Qiagen) pre equilibrated with Buffer A. The column was successively washed with Buffer B (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0, 8 M urea and 20 mM imidazole), Buffer C (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0 and 20 mM imidazole), Buffer D (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0, 20 mM imidazole and 10% glycerol) and Buffer E (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0, 20 mM imid-

azole, 10% Glycerol and 300 mM NaCl). Finally, the recombinant protein was eluted with Buffer F (100 mM sodium phosphate pH 8.0, Tris-HCl pH 8.0, 300 mM imidazole and 10% glycerol and 300 mM NaCl) in a refolded state.

### Characterization of truncated SIRT1

#### Western Blotting

Purified protein along with human serum sample was separated by SDS PAGE and transferred onto PVDF membrane (MDI). Membrane was then blocked with 3% Bovine Serum Albumin (Bio Basic Inc.) prepared in TBS1 (150 mM NaCl and 10 mM Tris.HCl pH 7.5) for 2 hrs followed by overnight incubation in 1:500 rabbit antihuman SIRT1 polyclonal antibody (Enzo Life Sciences). The blot was then washed with TBS-T (500 mM NaCl, 20mM Tris HCl pH 7.5 and 0.05% Tween) 3 times for 10mins and incubated with 1:5000 HRP (Horse Radish Peroxidase) conjugated goat secondary antibody (Genescript). The membrane was washed as above with TBS-T and visualized using Enhanced Chemiluminescent system (Thermo Scientific).

#### Surface Plasmon resonance

All SPR measurements were performed at 25°C using the BIAcore-2000 apparatus (Pharmacia Biosensor AB, Uppsala, Sweden) which is a biosensor-based system for real time specific interaction analysis. The IgG SIRT1 of human origin (ENZO Biotechnology, CA) was immobilized on the CM5 sensor chip using the amine coupling kit (Pharmacia Biosensor AB, Sweden). The running buffer used was 10 mM HBS-EP (10 mM HEPES pH 7.4, 150 mM Sodium Chloride, 3 mM EDTA, 0.005% Polysorbate 20) containing 0.005% surfactant P20. The dextran on the chip was equilibrated with running buffer and was activated with an equal volume (110  $\mu$ l) of EDC (N-ethyl-N'-3 diethylaminopropyl carbodiimide) (15 mg in 200  $\mu$ l of water) and NHS (N-hydroxysuccinimide) (2.3 mg in 200  $\mu$ l of water) mixture. This mixture was passed at a flow rate of 5  $\mu$ l/min for 15 mins. Then 250  $\mu$ l of antibody with 100  $\mu$ l in 10 mM sodium acetate (pH 4.7) was injected at a flow rate of 5  $\mu$ l/min and the unreacted groups were blocked by ethanolamine (pH 8.5).

Six different concentrations of the purified human truncated SIRT1 (0.62, 3.12, 6.25, 18.75, 31.25, 62.25 ng) were passed over the immobilized SIRT1 antibody. As the analyte (pure human truncated SIRT1) binds with the immobilized anti-IgG SIRT1 (ligand) over the CM5 sensor chip, an increase in SPR signal (expressed in response units, RU) was observed. The binding of the pure human truncated SIRT1 was monitored by the change in RU values of the sensogram and the corresponding RUs were obtained.

#### Mass Spectrometry Analysis

The purified protein band was excised from the gel and then subjected to in gel reduction, alkylation and tryptic digestion. The MS/MS was used to determine the mass and sequence of the protein. Each of the peptide was used to BLAST search to confirm that the protein identified by Mascot search programme was the only relevant match in the non redundant protein database for a particular peptide sequence and statistically significant hits were recorded together with the number of peptides and percentage coverage of the protein.

**Activity assay for SIRT1**

*Synthesis of Acetylated N terminal tail of histone H3(AcH3)*  
 Acetyl lysine peptide based on the sequence of N terminal tail of histone H3(AcH3) having sequence H<sub>2</sub>N-KSTGGK(COCH<sub>3</sub>) APRKQ-OH was synthesised by solid phase peptide synthesis using automated peptide synthesizer (PS3 Protein technology, USA) using Fmoc and Wang resin (G.L. Biochem, China) chemistry. The solvent used for synthesis was DMF (dimethylformamide). HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) was used as an activator of the Fmoc amino acids. Fmoc was deprotected by 20% piperidine and Wang resin was cleaved by TFA (trifluoroacetic acid). The peptides were precipitated from dry ether.

**Purification of Nicotinamidase**

*E.coli* BL21(DE3) cells harboring plasmid pPNC2 which encodes nicotinamidase protein were allowed to grow until absorbance at 600 nm reached 0.6 and then induced with 1 mM IPTG. Harvested cells were dissolved in buffer1 (20 mM sodium phosphate buffer pH 7.5, 500 mM NaCl and 20 mM imidazole), sonicated and the cell free extract was loaded on Ni-NTA resin (Qiagen) pre equilibrated with buffer1, followed by washing with the same buffer and finally the protein was eluted with buffer2 (20 mM sodium phosphate buffer pH 7.5, 500 mM NaCl and 500 mM imidazole). [24]

*Enzyme coupled assay*

Reaction mixture for the coupled enzymatic assay consisted of 3.3 mM AcH3, 3 mM NAD<sup>+</sup>, 2.4 mM NAD(P)H (Nicotinamide adenine dinucleotide phosphate), 13 mM DTT (Dithiothreitol), 13.7 mM αKG (αKetoglutarate), 2 units of GDH (Glutamate dehydrogenase) and 2 μM Nicotinamidase. All assay components except SIRT1 were incubated until absorbance at 340 nm gets stabilized and the reaction was initialized by the addition of truncated SIRT1 protein [25].

**Results**

**Bioinformatics Analysis**

The sequence of a 4110 bp mRNA of full length human SIRT1 was obtained from Gen Bank under accession no. NM\_012238 which contain an ORF of 2244 bp encoding a protein of 747 amino acid residues. Since the catalytic core of full length human SIRT1 spans a region of 244-498 amino acid residues, forward and reverse primers were designed to amplify a1260 bp fragment starting 577 bp downstream of the original ORF initiation site encoding amino acid sequences from 193-612 residues (Fig1). The gene bank accession No. is JQ768366. The deduced protein was found to be 47 kDa having a pI value of 5.1 by using Bioinformatics tool.

**Cloning**

Truncated SIRT1 gene was amplified using Pfu polymerase and the PCR product was found to be of 1260 bp. The purified PCR product was then cloned into pGEMT easy vector successfully and sequencing results showed that there was no nucleotide error in the truncated SIRT1 gene.

Prokaryotic expression vector pET28a<sup>+</sup> which possesses a high stringency T7 promoter and an N- terminal His Tag has been recognized as one of the most powerful tool for producing recombinant proteins in *E. coli*. The vector also possesses a thrombin cleavage site. The truncated SIRT1 gene was directionally sub-

cloned into pET28a<sup>+</sup> and transformed into *E.coli* BL21(DE3) cells.

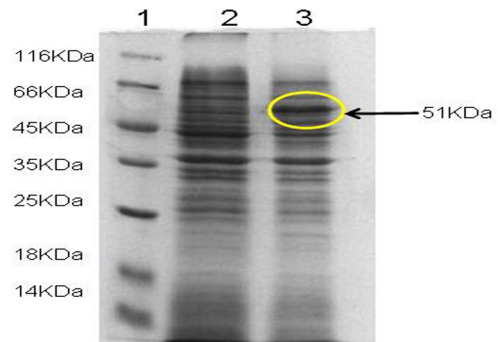
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1 atgattggcacagactcctgcagaacaattcttaagatttattgccc
  M I G T D P R T I L K D L L P
46 gaaacaatcctccacctgagttggatgatgacactgtggcag
  E T I P P P E L D D M T L W Q
91 attgttataatcctcttcagAACCCAAAAAGAAAAAAGA
  I V I N I L S E P P K R K K R
136 aaagatattaatacaattgaagatcctgtgaattactgtcaagag
  K D I N T I E D A V K L L Q E
181 tgcAAAAAattatagttcttaactggagctggggtgtctgttca
  C K K I I V L T G A G V S V S
226 tgtggaatcctgactcaggctcaaggatgttattatgctcgc
  C G I P D F R S R D G I Y A R
271 cttgctgtagactccccagatcttcagatcctcaaggatgttt
  L A V D F P D L P D P Q A M F
316 gatattgaatatccagaaagatcccaagaccatcttcaagttt
  D I E Y F R K D P R P F F K F
361 gcaaaagaaatatactcggcaactccagccatctctctgtcac
  A K E I Y P G Q F Q P S L C H
406 aaattcatagcctgtcagataaggaagaaactactctgcac
  K F I A L S D K E G K L L R N
451 tataccagaacatagacacgctggaacaggttggggaaatccaa
  Y T Q N I D T L E Q V A G I Q
496 aggataattcagtgatcgtggttcttggcaacagatcttgccctg
  R I I Q C H G S F A T A S C L
541 atttgaatacaaaagttgactgtgaagctgtacgaggactctt
  I C K Y K V D C E A V R G A L
586 tttagtcaggtagttcctcctgagtgctgagtgccagctgagaa
  F S Q V V P R C P R C P A D E
631 ccgcttgctatcatgaaaccagagattgtgtttttggtgaaat
  P L A I H K P E I V F F G E N
676 ttaccagaacagtctcatagagccatgaagatgacaagatgaa
  L P E Q F H R A R A G Y D K D E
721 gttgacctcctcattgttattgggtcttccctcaaagtaagacca
  V D L L I V I G S S L K V R P
766 gtacactaatcccaagttccatcccccagtgctcctcagata
  V A L I P S S I P H E V P Q I
811 ttaataatagagaactttgctcctcctgctatttggatgtagag
  L I N R E P L P H L H F D V E
856 ctcttggagactgtgatgcatcaatcaatgattgtgcatagg
  L L G D C D V I I N E L C H R
901 ttagtggtggaatagccaaacttggctgtaaacctgaaagctt
  L G G E Y A K L C C N P V K L
946 tcagaataactgaaaaaacctccacgacacacaaagaattggct
  S E I T E K P P R T Q K E L A
991 tatttgcagaggtgccaccacactcttcatgtttcagaagac
  Y L S E L P P T P L H V S E D
1036 tcaagttaccagaagaactcaccaccagattcttcagtgatt
  S S S P E R T S P P D S S V I
1081 gtcacacttttagaccaagcagctaaagtaagatgatttagat
  V T L L D Q A A K S N D D L D
1126 gtgtctgaatcaaaaggttgtatggaagaaaaaacacaggaagta
  V S E S K G C M E E K P Q E V
1171 caaactctaggaatgttgaagattgtgtaacagatggaaat
  Q T S R N V E S I A E Q M E N
1216 ccggatttgaagaatgtgtggtcttagtactggggagaaaaatgaa
  P D L K N V G S S T G E K N E
    
```

**Fig. 1-** The cDNA sequence and deduced 193-612 amino acid residues of human truncated SIRT1

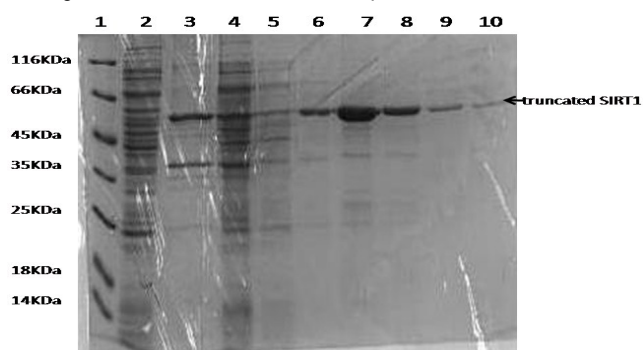
**Expression and Purification**

The expression of truncated gene was induced with 1 mM IPTG. Induced and uninduced cultures were compared by SDS PAGE and a specific band of about 51 kDa corresponding to truncated SIRT1 protein was detected in induced culture (Fig 2).



**Fig. 2-** Expression of human truncated SIRT1 in *E. coli* BL21 cells. Lane 1: molecular weight marker, Lane2 and 3: uninduced and induced cells respectively

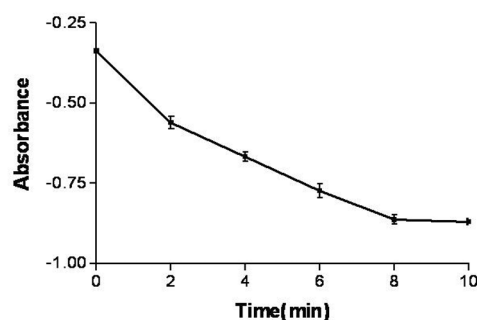
In order to obtain highly purified recombinant protein the inclusion bodies were solubilised in 8M urea and passed through Ni-NTA resin. The purity of the eluted recombinant protein was analyzed by SDS PAGE which detected a single band of about 51 kDa (Fig 3). The protein sequence obtained of molecular weight 47 kDa showed 51 kDa band in SDS PAGE due to the presence of fused His tag along with extra amino acid residues provided by the vector. Recombinant truncated SIRT1 was produced in large quantities, greater than 5% of the total cell proteins.



**Fig. 3-** Purification of human truncated SIRT1 by Ni-NTA column: Lane1: Molecular weight marker, Lane2-5:flow through and washings, Lane 6-10: refolded purified protein

#### Western Blot

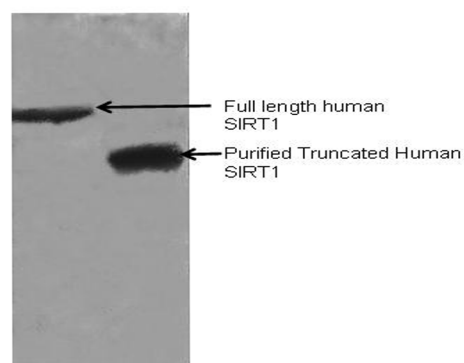
Purified truncated SIRT1 which reacted with rabbit anti SIRT1 polyclonal antibody was apparent as a single band. To compare the full length and truncated protein we used human serum sample and found that the molecular weight of full length human SIRT1 was of around 81kDa while the truncated protein was of only 51 kDa (Fig 4).



**Fig. 4-** Western blot showing the specificity of human truncated SIRT1 compared to SIRT1 present in human serum

#### Truncated SIRT1 assessment by SPR.

The SPR signal for immobilization of antibody of SIRT1 was found to be 14908.5 RU where 1 RU corresponds to immobilized protein concentration of 1 pg/mm<sup>2</sup>. Six different amounts of pure recombinant SIRT1 in HBS-EP buffer were passed over the immobilized SIRT1 anti body and the RU obtained were 14918.4, 15021.1, 15115.5, 15473.6, 15768.3 and 16982.0 RU. The curve was plotted with RU obtained from the sensogram with different concentrations of pure truncated SIRT1 as mentioned above. The binding of the ligands i.e. truncated SIRT1 were in the linear range shown in Fig 5. The RU increased linearly as the concentration of truncated SIRT1 increases.



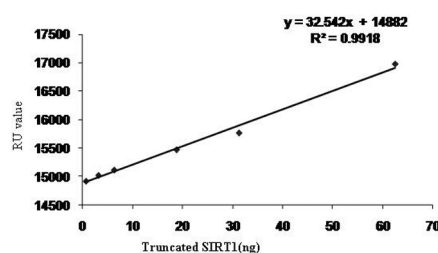
**Fig. 5-** RU values obtained by passing six different amounts (0.62 ng, 3.12 ng 6.25 ng, 18.75 ng, 31.25 ng and 62.25 ng) of truncated SIRT1 over immobilized antiSIRT1 antibody

#### MASS Spectrometry

The purified protein band was excised from gel and identified through Mass Spectrometry. The trypanized fragments were analyzed through a mascot search program from matrix science shows candidate proteins as SIRT1 and a score of 116 based on probability analysis. The score of 116 for an MS/MS match in Mascot search depends upon the absolute probability that the selected match between reported data and the database sequence is a random event.

#### Enzyme Activity

The activity assay was performed as described by Smith et al in which the rate of nicotinamide formation was measured using a coupled enzyme system with nicotinamidase and glutamate dehydrogenase. Nicotinamidase hydrolyses nicotinamide to nicotinic acid and ammonia. Glutamate dehydrogenase then converts ammonia,  $\alpha$ -ketoglutarate, and NAD(P)H to glutamate and NAD(P)<sup>+</sup>. NAD(P)H oxidation/consumption is measured spectrophotometrically at 340 nm. A linear response was obtained from the coupled enzyme reaction (Fig 6). Through this assay it was established that the purified truncated protein possess histone deacetylase activity.



**Fig. 6-** Activity assay of human truncated SIRT1: Linear response due to the consumption of NAD(P)H with time.

#### Discussion

The first gene discovered in the Sirtuin family was Sir2 from yeast, is able to extend the life span in various organisms. Seven human homologous of sirtuins (SIRT1-7) which share the catalytic domain with Sir2 have been characterized [26, 27]. The anti ageing effects of human homologous of sirtuins are also seen by animal and human associated studies. SIRT1, the most studied of 7 sirtuin family members, is a key mediator of the pathways down-



stream of calorie restriction, dietary regimen that is known to delay the onset of ageing and reduce the incidence of age related diseases. SIRT1 has been proved to be a potential therapeutic target for age associated diseases.

Since the catalytic domain of full length human sirtuin consist of 255 conserved amino acids (244-498 aa), we have cloned and expressed a protein from 193 to 611 amino acids residues to keep the activity intact in expressed protein. The primers were designed based on the sequence of human SIRT1 mRNA sequence which was obtained from NCBI (NM\_012238) of 4110 bp. The cloned gene includes an ORF of 1260 bp encoding 420 amino acids residues.

The protein was characterized by mass spectroscopy, SDS PAGE and Western Blot was found to be of 51 kDa and pI value of 5.1. The specificity of protein was identified by using its specific antibody by western blotting and SPR. In SPR the sensogram increases linearly as the mass of the SIRT1 increases over the antibody.

This sequence shows high similarity with other species' SIRT1. The amino acid sequence of truncated SIRT1 sequence shared 99% identity to SIRT1FL\_human, 97% identity to *Sus scrofa*, 94% identity to *Bos taurus*, 93% to *Cricetulus griseus*, 93% to *Mus musculus*, 94% to *Oryctolagus cuniculus*, 92% to *Callithrix jacchus*, 91% to *Ailuropoda melanoleuc*, 93% to *Mustela putorius furo*, 51% to *Drosophila melanogaster* and 41% to *Caenorhabditis elegans* (Table 1). The Fig. 7 shows the high homology of truncated SIRT1 with the conserved catalytic domain of other mammalian SIRT1.

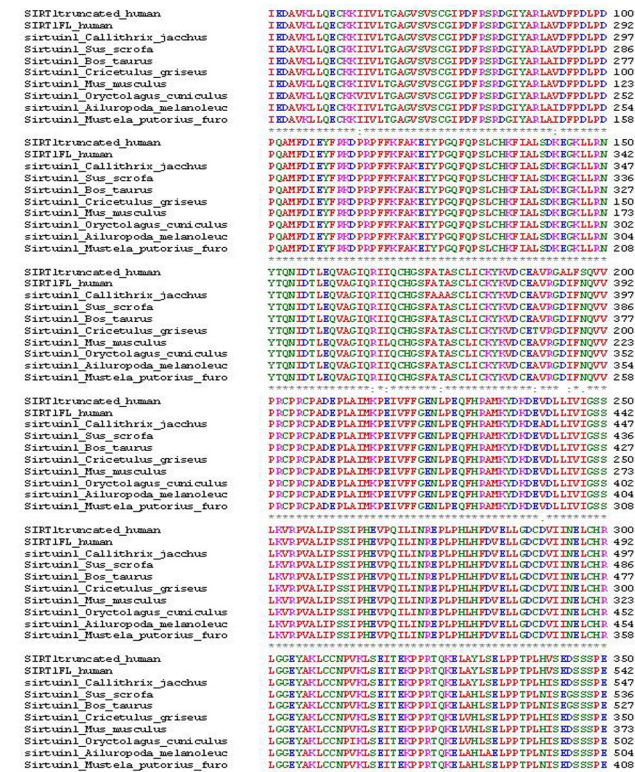


Fig. 7- The sequence alignment of catalytic domain of human truncated SIRT1 with other mammalian SIRT1

Table-1- The sequence similarity of human truncated SIRT1 with sirtuin of other species

Species name	Gen bank accession no.	Percentage identity
Homo sapiens	NP_036370.2	99
Callithrix jacchus	XP_002756358.1	92
Sus scrofa	NP_001139222.1	97
Bos taurus	NP_001179909.1	94
Cricetulus griseus	EGW03730.1	93
Mus musculus	AAI52315.1	93
Oryctolagus cuniculus	XP_002718507.1	94
Ailuropoda melanoleuc	XP_002913805.1	91
Mustela putorius furo	AES06615.1	93
Drosophila melanogaster	NP_477351	51
Caenorhabditis elegans.	CBK19468.1	41

The enzymes that produce the nicotinamide from the catabolism of NAD<sup>+</sup> are the mediator of many physiological processes [28]. Sirtuin protein used NAD<sup>+</sup> as a co-substrate during catalysis for deacetylation to remove the acetyl group from the acetyl lysine residues of protein forming deacetylated protein, o-acetyl-ADP-ribose and nicotinamide. Sirtuin use NAD<sup>+</sup> to form one molecule of nicotinamide for every lysine residue deacetylated. The cloned truncated SIRT1protein in the present study binds with both substrates Ach3 and cosubstrate NAD<sup>+</sup> to produce nicotinamide.

**Conclusion**

It can be concluded that the truncated sirtuin 1 of 420 amino acids which is homologous to full length SIRT1 can be easily expressed in *E. coli*. The protein is purified and refolded in active form and binds to both substrate and co substrate to deacetylate the acetylated protein. It can be used for the in vitro study for the development of new therapeutic agent.

**Acknowledgements**

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