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Comparative deacetylase activity of wild type and mutants of SIRT1

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ABSTRACT

SIRT1, human ortholog of yeast SIR2 protein, deacetylates histones and several other transcription factors. Recently, SIRT1 has emerged as a drug target for treating age related diseases, type II diabetes, neurodegeneration, inflammation and cancer. Here, we have optimized production of functionally active wild type full-length SIRT1 protein and its N-terminal deleted mutants. In a comparative study, we found that the region containing 192–208 amino acids towards the N-terminus is critical for right conformational folding of the protein to retain its deacetylase activity. The EC_{50} and IC_{50} values obtained with standard modulators showed that the SRT_{748} & SRT_{556} can deacetylate substrate and are activated by resveratrol, whereas, deacetylase activity of all the other deletion mutants (SRT_{540}, SRT_{532}, SRT_{507} and SRT_{503}) was lost. We further report that the peptide substrate K_m for SRT_{748} (70 ± 5.2 μM) was comparable to SRT_{556} (93 ± 5.4 μM). The K_m for NAD^+ substrate was 176 & 274 μM for SRT_{748} and SRT_{556}, respectively. Similar substrate affinity studies demonstrate that either of the protein (SRT_{748} or SRT_{556}) can be utilized for screening SIRT1 modulators. We have also examined critical regions in SIRT1 required for deacetylase activity as well as kinetic analyses of SIRT1 proteins.

Introduction

The sirtuin (silent information regulator—SIR2) family of genes is a highly conserved group present in the genomes of organisms ranging from archaebacteria to eukaryotes. The first sirtuin gene SIR2 was discovered in yeast after which seven more sirtuins, SIRT1–SIRT7, were discovered in mammals [1]. Among these diverse sirtuins present in mammals, SIRT1 is the most extensively studied and closest homologue of yeast SIR2.

Sirtuin deacetylates acetyl lysines on various histone or non-histone protein substrates [2]. Histone acetylation and deacetylation mechanism functions as an essential switch that turns gene transcription on and off. Acetylation neutralizes the positive charges of histone, resulting in their low affinity for negatively charged DNA, which promotes the formation of an "open" structure for proteins to bind to DNA and makes them transcriptionally active [3]. Earlier studies have established that the transcriptionally active areas of the genome are hyperacetylated, whereas histones within the silent regions of chromatin are known to be hypoacetylated [4].

In yeast, SIR2, has been linked to cellular senescence and also promotes longevity [5]. Increasing the levels of SIRT1 homologues in fruit flies [6] and roundworms [7] was also found to extend lifespan. In mammals, SIRT1 deacetylates key transcription factors and co-factors, such as p53 [8], FOXO (forkhead) proteins [9], and nuclear factor-kB (NF-kB) [10], thereby affecting crucial cellular and metabolic pathways involved in stress response. Recently, SIRT1 over expression has been linked to the development of malignant phenotype [11,12]. Sirtuins have been shown to regulate critical steps in ageing, metabolism and cancer which make them attractive targets for the development of new therapeutics [13].

Activity of sirtuins can be modulated by small molecule drugs. In 2003, a study by Howitz et al. [14] demonstrated that resveratrol increases deacetylation activity of SIRT1 which in turn stimulates other SIRT1 mediated cellular processes [6,14,15]. A recent report has shown that SIRT1 activation inhibits transcription of the gene phosphoenolpyruvate carboxykinase (PEPCK-C) by deacetylating

Abbreviations: NAD^+, nicotinamide adenine dinucleotide; K_m, Michaelis constant; SIR2, silent information regulator; NaCl, sodium chloride; DTT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; EC_{50}, effective concentration; V_{max}, maximum velocity; IPTG, isopropyl β-D-1-thiogalactopyranoside; CBBR, coornassie brilliant blue R-250; SDS–PAGE, sodium dodecyl sulphide–polyacrylamide gel electrophoresis; GST, glutathione S-transferase; IC_{50}, half maximal inhibitory concentration; PEPCK-C, phosphoenolpyruvate carboxykinase; HNF, hepatic nuclear factor 4α

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hepatic nuclear factor (HNF) 4α, thus leading to a longer lifespan [16]. On the contrary, cancer cells might develop resistance to chemotherapy by over expressing SIRT1 [17]. These authors observed that the resistant tumor cells can be sensitized to the drug by downregulating SIRT1 expression. Therefore it is highly likely that by developing SIRT1 inhibitors drug-resistance in cancer cells can be overcome [17].

Screening of small molecule modulators of sirtuin requires bulk quantity of purified recombinant enzyme. Production of in-house recombinant SIRT1 protein was initiated and further identification of protein segment that contributes to its deacetylase activity and activation was determined. Based on homology modeling of SIRT1 with known SIRT2 crystal structure, three important regions have been identified in SIRT1 protein: N-terminal region (1–243), catalytic domain (244–467), and C-terminal region (499–747) [18]. Another study on different N- and C-terminal deleted SIRT1 proteins have shown that region containing 183–225 amino acids may have putative allosteric binding site, where an activator binds and improves enzyme substrate affinity [19]. Recently, Sasaki et al. [20] have shown that key amino acids in the N-terminal and C-terminal region are phosphorylated. It was observed that a decline in phosphorylation of SIRT1 led to a decrease in deacetylation activity of the protein.

The objectives of the current study were; (1) to produce full-length SIRT1 protein (SRT_{748}) and a series of N-terminal deleted proteins (SRT_{556}, SRT_{540}, SRT_{532}, SRT_{507}, SRT_{503}) in bacterial expression system, (2) determine important regions in SIRT1 protein required for deacetylase activity, (3) kinetic characterization of functional SIRT1 protein and determining mechanism of activation with resveratrol.

Materials and methods

PCR amplification and cloning of SIRT1: Full length and partial cDNAs of SIRT1 were cloned into pGEX4T2 vector (GE Healthcare) after amplification with gene specific primers from clone obtained from OriGene (OriGene Technologies, Rockville, MD). The primer sequence for amplification of SRT_{556} cDNA was (5′-CGCGGATCCATTGGCACAGATCCTCGAACAATTCT-3′) for forward primer and (5′-CCGCTCGAGCTATGATTTGTTTGATGGATAGTTCATGTC-3′) for reverse primer. SRT_{748} was amplified using forward primer (5′-CGCGGATCCGCGGACGAGGCGGCCCT-3′) and reverse primer was same as that of partial gene. The primers were designed especially for cloning into pGEX4T2 vector (GE Healthcare) at *BamHI* & *XhoI* sites for SRT_{556} and at *BamHI* and *NotI* sites for SRT_{748}. The cloned cDNA of human SIRT1 was confirmed with restriction mapping and sequencing.

Induction of recombinant SIRT1 protein: The SIRT1 recombinant plasmids were transformed into *Escherichia coli* strain BL21 RIPL (InVitrogen). Single colonies were selected and grown overnight in 2 ml Luria Bertani medium containing ampicillin (100 μg/ml) at 37 °C. From these overnight cultures, 1 ml was transferred to 500 ml of Luria–Bertani medium containing ampicillin (100 μg/ml). The cells were grown at 37 °C for approx. 3 h until the OD_{600} was between 0.5 and 0.6. To induce protein expression, Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added and the cells were grown at 37 °C for a further 6 h. Cells were collected from a 50 ml culture by centrifugation at 4000*g* for 5 min. The pellet was resuspended and lysed by incubation in 2.5 ml of lysis buffer (10 mM Tris–Cl, 0.15 M NaCl, 10 mM DTT, 10% glycerol, 1% Triton-X) per 50 ml of culture, with gentle mixing at room temperature. The resuspended material was lysed by 6 cycles of freeze and thaw and then centrifuged at 27,000*g* for 10 min to remove cell debris. The soluble cell lysate was stored at −20 °C until required for further purification.

Purification of SIRT1 protein: Purification of N-terminal GST-tagged recombinant SIRT1 protein was carried out on Econo columns (Bio-Rad) packed with GST resin (Novagen) according to manufacturer's specifications. The column was equilibrated by incubation with binding/wash buffer (10 mM Sodium phosphate, 140 mM NaCl, 2.7 mM KCl pH 7.4). Following equilibration, lysate was added to the column. Bound protein was washed thoroughly with wash/binding buffer. The recombinant protein was eluted from the column with 6 ml of elution buffer (50 mM Tris–HCl, 20 mM reduced Glutathione pH 8.1) and 1 ml fractions were collected. Elution fraction were analyzed by SDS/PAGE for presence of purified recombinant protein. The protein concentrations were determined by the Bradford procedure (Bio-Rad Laboratories) using bovine serum albumin as the standard. Purified protein was confirmed with Western blotting. The proteins separated on 10% SDS/PAGE were transferred to nitrocellulose membrane. The membrane was incubated with rabbit polyclonal SIRT1 IgG at 1:1000 dilution followed by incubation with secondary goat anti-rabbit polyclonal IgG HRP conjugated at 1:2000 dilution. SIRT1 specific protein was detected using chemiluminescence's kit (Pierce).

Functional assay for SIRT1: The activity of purified SIRT1 was tested with Biomol SIRT1 Fluorescence Assay kit (AK-555) according to manufacturer's instructions. The peptide used in the assay comprises of amino acids 379–382 of human p53 (Arg-His-Lys-Lys (Ac)). The fluorescence signal is generated in proportion to the amount of deacetylation of the lysine corresponding to Lys-382, a known in vivo target of SIRT1 activity. NAD^+-dependent deacetylation of the substrate by recombinant human SIRT1 sensitizes it to Developer II, which then generates a fluorophore. The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. In the assay, SIRT1 deacetylates an acetylated SIRT1 specific peptide and the enzyme activity was measured as increase in fluorescence. Upon activating SIRT1 protein with resveratrol, an increase in fluorescence was measured. Inhibition of enzyme with suramin inhibitor (Biomol) was measured as decrease in fluorescence. EC_{50} & IC_{50} graphs for resveratrol and suramin was generated using GraphPad Prism software. K_m & V_{max} of the purified enzymes were also calculated keeping one substrate constant and varying another substrate. The graphs were generated in GraphPad Prism software. The error bars indicate mean ± SEM of at least three experiments performed in triplicate.

Results

Expression and purification of wild type and deletion mutants of recombinant hSIRT1

Human wild-type SIRT1 amplified PCR product and its various deletion mutants (Fig. 1A) were fused in frame at N-terminal with GST-tag into pGEX4T2 expression vector. The SIRT1 constructs differentiated towards the N-terminal of protein, where amino acid deletions were made. All the recombinant SIRT1 plasmids were transformed into DH5α cells. The cloned cDNA sequences were verified by restriction mapping and sequencing. For expression study, the recombinant SIRT1 cDNAs were transformed into RIPL strain of BL21 cells. Expression was induced with 1 mM IPTG at 37 °C for 6 h. Expression analysis of supernatant and cell pellet showed that all the proteins are expressed in the soluble form. The cell lysates were analyzed for recombinant protein expression by Western blot using SIRT1 specific antibodies. Expected molecular weight of the proteins were observed in the Western blot of cell lysates as 108.3 kDa (SRT_{748}), 87 kDa (SRT_{556}), 85 kDa (SRT_{540}), 85 kDa (SRT_{532}), 82 kDa (SRT_{507}), 81 kDa (SRT_{503}) (Fig. 1B). We ob-

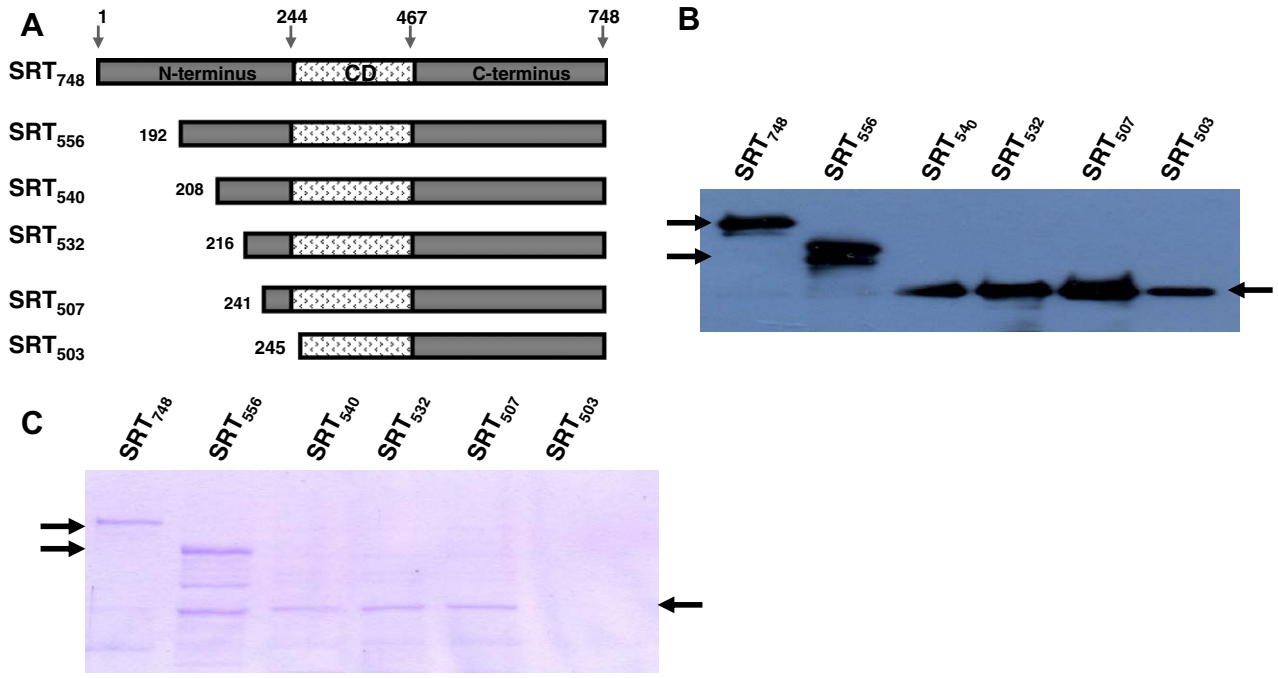

Fig. 1. (A) A series of N-terminal deletion mutants of SIRT1 that characterize important region involved in deacetylase activity. (B) Western blot analysis of full-length SIRT1 and all deletion mutants of SIRT1 expressed in BL21 RIPL stain of *E. coli* probed with polyclonal SIRT1 antibody. (C) SDS–PAGE analysis of recombinant SIRT1 protein, full length and series of mutants, expressed in *E. coli* purified through affinity chromatography.

served that SRT_{748} and SRT_{556} were better expressed when compared to SRT_{540}, SRT_{532}, SRT_{507}, and SRT_{503}.

Cell pellets from 50 ml culture of all the SIRT1 proteins were lysed for protein purification. The cell growth and lysis protocols were carried out as described in the methods. The proteins were purified with affinity chromatography and eluted with 20 mM Glutathione. The purified proteins were analyzed on SDS–PAGE stained with coornassie brilliant blue R-250 (CBBR) and confirmed with Western blotting. Expected bands of all the SIRT1 proteins were observed on SDS–PAGE (Fig. 1C).

Deacetylase activity of purified recombinant SIRT1 proteins

A functionally active purified recombinant SIRT1 protein is required for screening of SIRT1 modulators in drug discovery. In this study, we first set out to understand deacetylase activity of all SIRT1 recombinant clones (SRT_{748}, SRT_{556}, SRT_{540}, SRT_{532}, SRT_{507}, SRT_{503}) on *Fluor de Lys* (Biomol) SIRT1 peptide substrate. Same concentrations (125 ng) of all recombinant purified enzymes were tested for enzymatic deacetylation reaction. We observed highest deacetylation of SIRT1 peptide by SRT_{748} followed by SRT_{556}. Whereas, all other deleted purified proteins did not show any deacetylase activity (Fig. 2A). Deletion mutant's data confirm that region 192–208 of amino acids is critical for correct refolding of protein in order to retain its deacetylase activity as observed in SRT_{540}. A linear dose-dependent deacetylation of *Fluor de Lys* SIRT1 substrate peptide was observed for SRT_{748} & SRT_{556} proteins (Fig. 2B). Retention of deacetylating activity in SRT_{748} and SRT_{556} suggests that both the enzymes deacetylate SIRT1 substrate and either one can be utilized in the drug discovery process.

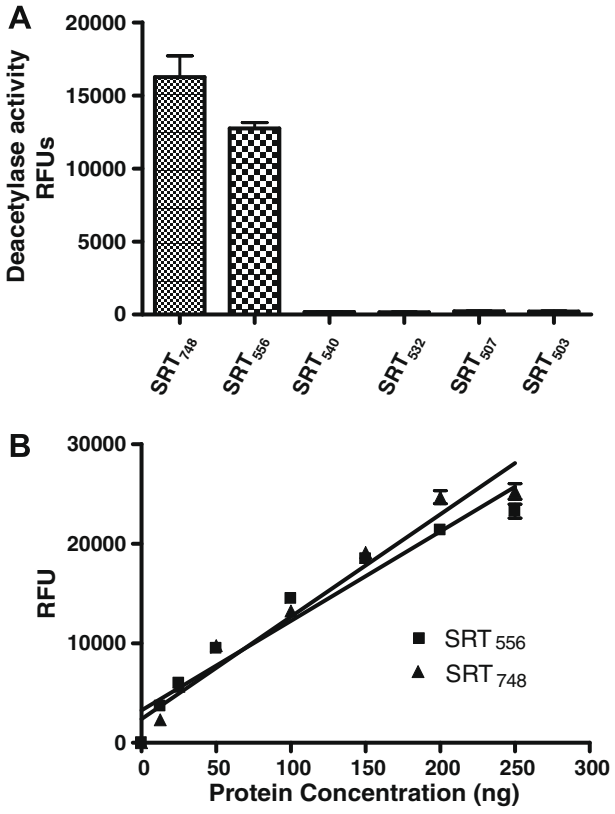

Fig. 2. (A) Deacetylase activity of SRT_{748}, SRT_{556}, SRT_{540}, SRT_{532}, SRT_{507}, and SRT_{503} on *Fluor de Lys* SIRT1 substrate (Biomol). (B) Linear dose relationship curve for functionally active SRT_{748} and SRT_{556}.

Kinetic characterization of functional SIRT1 proteins

Before evaluating SIRT1 modulators, it is necessary to examine the recombinant SIRT1 protein for retention of its activation and inhibition properties. The purified recombinant enzymes, SRT_{748} and SRT_{556}, were tested for inhibition with SIRT1 specific inhibitor-suramin. Both SIRT1 enzymes showed similar IC_{50} of 2 ± 0.07 & 1 ± 0.04 μM with suramin (Fig. 3A). Upon activation 1.6- and

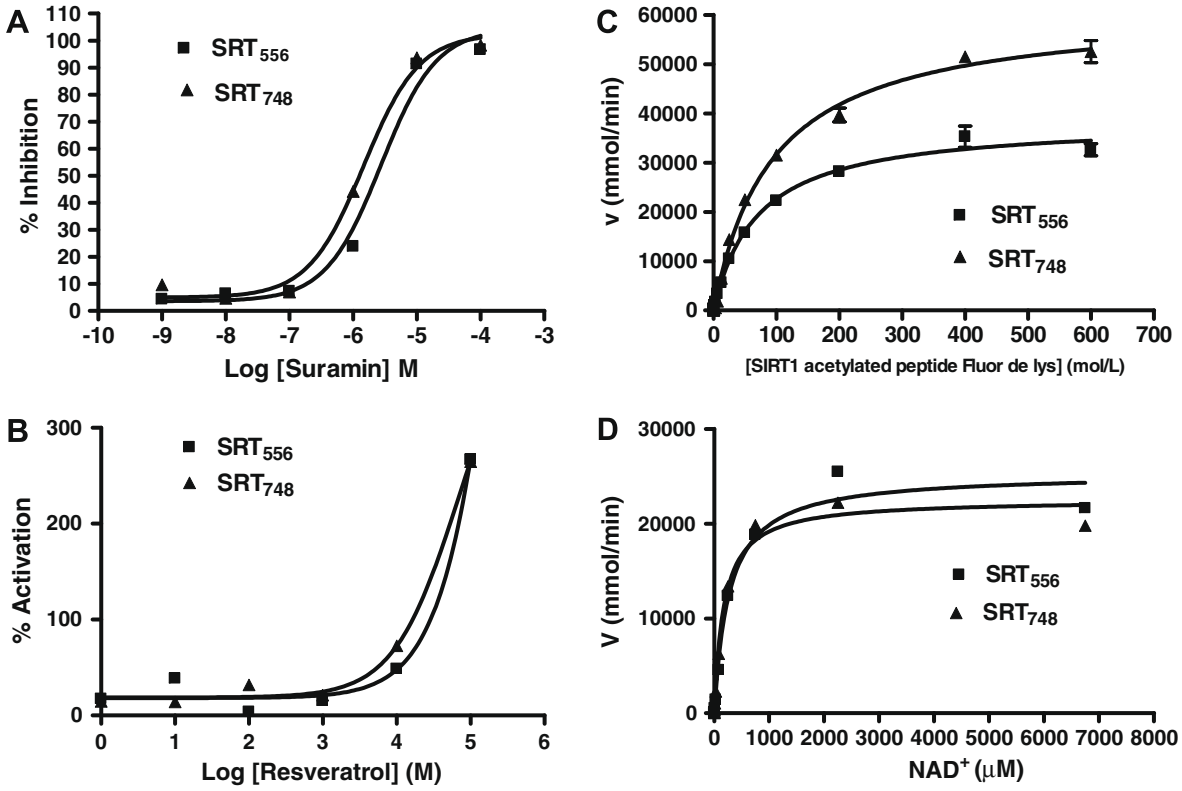

Fig. 3. (A) Suramin inhibition of SRT_{748} and SRT_{556}. (B) Resveratrol activation of SRT_{748} and SRT_{556}. (C) K_m & V_{max} of SRT_{748} and SRT_{556} with varying concentrations of *Fluor de Lys* SIRT1 substrate. (D) K_m & V_{max} of SRT_{748} and SRT_{556} with varying concentrations of NAD^+ (μM).

1.43-fold activation for $SIRT_{748}$ and $SIRT_{556}$ was observed at 10 μM concentration of resveratrol. Varying concentrations of resveratrol showed an increase in activation and both forms of the enzymes achieved maximum activation of ~266% at the highest tested concentration of resveratrol (100 μM) (Fig. 3B). The EC_{50} obtained for SRT_{556} and SRT_{748} was 5.4 & 10 μM, respectively. Similar activation and inhibition profiles of SRT_{748} & SRT_{556} proteins corroborated our observation that either one can be utilized for the screening of SIRT1 modulators.

To further characterize the enzyme, a detailed kinetic analysis was performed whereby K_m was determined for interactions of peptide and co-factor NAD^+ at the catalytic site. The *Fluor de Lys* SIRT1 peptide concentrations were varied ranging from 0.1 to 600 μM with a fixed concentration of SIRT1 (125 ng/well) and NAD^+ (250 μM). As shown in Fig. 3C, the K_m of SRT_{748} for *Fluor de Lys* SIRT1 peptide as determined from Lineweaver Burk plot was 70 ± 5.2 μM. Slightly lower K_m of 93 ± 5.4 μM was obtained for SRT_{556} with the same *Flour de Lys* peptide. With increasing dose of *Fluor de Lys* SIRT1 peptide substrate, an equal increase in the velocity of reaction was observed for SRT_{748} and SRT_{556} suggesting that both the enzymes are comparable in terms of peptide binding (Fig. 3C). When NAD^+ was varied K_m for SRT_{556} & SRT_{748} was 274 & 175 μM, respectively (Fig. 3D). Similarly, K_m observed for both peptide and NAD^+ substrate with two different enzymes showed that 1–191 amino acids towards the N-terminal are not critical for peptide affinity. These studies clearly established that the recombinant SIRT1, SRT_{748} and SRT_{556}, do not differentiate much on K_m value with *Fluor de Lys* SIRT1 peptide & NAD^+.

In order to evaluate whether affinity of SRT_{748} & SRT_{556} enzymes with its peptide substrate is improved upon resveratrol activation, the K_m and V_{max} of the proteins were further compared. Deacetylation of SIRT1 peptide at a range of peptide concentrations and a fixed concentration of NAD^+ was measured. With increasing concentration of resveratrol (10, 50, 100, 200 μM) the K_m for *Fluor de Lys* substrate decreased 4-fold for SRT_{556} and 3.5-fold for SRT_{748}. The maximal velocity, V_{max} of the deacetylation reaction remained similar by resveratrol activation (Table 1). Lowering of K_m with increasing resveratrol concentration confirmed that mechanism of SRT_{748} & SRT_{556} activation is by enhanced binding of peptide substrate with the SIRT1 protein.

Discussion

Sirtuins have been implicated in several diseases such as type 2 diabetes, cardiovascular disease, neurodegenerative, inflammation and cancer. SIRT1 activators are known for regulating diseases of aging and metabolic disorders [19], alternatively, inhibitors for the same are important in regulating cancer [12,21,22]. Screening for SIRT1 agonist and antagonist in drug discovery requires purified recombinant SIRT1 enzyme in bulk. Here we report, expression and purification of recombinant SIRT1 and characterization of its various N-terminal deletion mutants to determine the minimum fragment of SIRT1 protein required in drug discovery. Recombinant human proteins have been expressed in bacteria, yeast, mammalian [23] and baculovirus expression systems. Mammalian expression system is resource intensive and the yields of protein are low after purification, whereas baculovirus expression system is efficient compared to mammalian system, but still relatively expensive to produce proteins in bulk. These two expression systems are suitable for generating human recombinant proteins that require post translational modifications. If a post translational modification of eukaryotic protein is not a constraint then bacterial expression system is preferred whereby large amount of proteins can be produced in a short duration and more economically. A detailed discussion of biotechnology strategies for optimizing eukaryotic protein production in bacteria can be found in a recent

Table 1
Fluor de Lys fluorescence assay to determine mechanism of resveratrol activation of SRT_{748} and SRT_{556}. All assays were carried out in the presence of 125 ng of SIRT1 with 50 μM *Fluor de Lys* SIRT1, 250 μM NAD, in the presence of varying concentrations of resveratrol.

Resveratrol (μM)	200	100	50	10
SRT_{748} K_m	21.02 ± 4.8	21.96 ± 4.5	34.19 ± 2.9	74.08 ± 4.9
SRT_{556} K_m	26.42 ± 4	28.09 ± 4.8	37.33 ± 3.4	113.8 ± 6
SRT_{748} V_{max}	37,936 ± 2057	40,542 ± 1984	44,551 ± 985	40,505 ± 814
SRT_{556} V_{max}	38,508 ± 2215	43,038 ± 1855	44,854 ± 1086	44,725 ± 2059

review [24]. In the present study, bacterial expression of full-length SIRT1 protein, SRT_{748} and its other N-terminal deleted proteins (SRT_{556}, SRT_{532}, SRT_{507}, and SRT_{503}) was undertaken. The yield of purified SRT_{748} and SRT_{556} protein was approximately 1 mg per litre of bacterial culture. Protein yield of SRT_{540}, SRT_{532}, SRT_{507}, and SRT_{503} were significantly low compared to SRT_{748} and SRT_{556}. The lower yield of SRT_{540}, SRT_{532}, SRT_{507}, and SRT_{503} proteins could be due to an improper folding of the mutant.

Milne and Denu [18] based on molecular modeling studies with the crystal structure of known SIRT2 protein identified three critical domains in SIRT1 protein, including N-terminal, catalytic, and C-terminal domain. Catalytic domain of approximately 223 amino acids contain a NAD^+-dependent deacetylase activity. Our deletion mutant's analysis has revealed that the N-terminal sequence is also essential to achieve right protein confirmation and optimal deacetylase activity. We further observed that amino acids 192–208 are essential to achieve deacetylation activity and maintaining the activation of SIRT1 by resveratrol that probably defines the allosteric binding site. Previous studies indicated the amino acids 183–225 in N-terminal region may have putative allosteric binding site to which an activator binds and enhances enzyme–substrate affinity [19].

SRT_{748} and SRT_{556} are functionally and biochemically active, although the deacetylase activity is slightly lower in SRT_{556}. It was observed that percentage increase in the activity of both the proteins, SRT_{748} & SRT_{533}, was identical with an increasing concentration of enzyme. Further deletions after 191 amino acids resulted in a complete loss of activity. Recombinant SIRT1 (SRT_{748} and SRT_{556}) expressed in bacterial system maintained the correct conformation for the interaction with activator and inhibitor, as evident from our IC_{50} and EC_{50} studies for suramin and resveratrol, respectively. Suramin has been reported to be an inhibitor of SIRT1 with an IC_{50} value of 2.6 μM (Biomol International). K_m and V_{max} parameters for both the proteins exhibited similar kinetics over an increasing concentration of the peptide substrate.

In summary, our studies have clearly shown that the wild-type SIRT1 protein expressed in *E. coli* is properly folded, pharmacologically active and retains its deacetylase activity. The process is reliable, reproducible and the protein can be produced in large amount for HTS of potential SIRT1 activators and inhibitors.

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