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Capillary electrophoresis-based sirtuin assay using non-peptide substrates

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ABSTRACT

Sirtuins are NAD^+-dependent class III histone deacetylases, which catalyze the deacetylation of acetyl-lysine residues of histones and other protein substrates yielding the deacetylated protein, nicotinamide and 2′-*O*-acetyl-ADP-ribose. Two lysine amide derivatives containing dansyl (Dns) or 7-dimethylaminocoumarin (DMAC) residues, i.e. Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2, were synthesized and evaluated as substrates for human sirtuin 1. A CZE method with field amplified sample injection and a MEKC method with sweeping were established and validated for monitoring the deacetylation process of Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2, respectively. Deacetylation by sirtuin 1 was demonstrated for both of the substrates. The Michaelis–Menten constants, K_m, were 88.0 μM for Dns-K(Ac)-NH_2 and 42.9 μM for DMAC-K(Ac)-NH_2. The applicability of the methods was demonstrated using known sirtuin inhibitors. Resveratrol did not activate sirtuin 1 using the present CE-based enzyme assay. The results indicated that the lysine derivatives can be used in sirtuin assays instead of peptide substrates.

1. Introduction

Sirtuins are nicotinamide adenine dinucleotide (NAD^+)-dependent class III histone deacetylases catalyzing the deacetylation of acetyl-lysine residues of histones and other proteins yielding nicotinamide, the deacetylated protein and 2′-*O*-acetyl-ADP-ribose as products [1,2]. Seven sirtuin enzymes, SIRT1–SIRT7, have been identified in humans with diverse cellular locations and molecular targets regulating a wide range of biological processes such as proliferation, differentiation, metabolism, apoptosis and senescence [3–6]. SIRT1 as the most intensively investigated enzyme is considered a therapeutic target for metabolic, neurological, cardiovascular, renal and mitochondrial related diseases as well as cancer [4–6]. Thus, research efforts have been directed to understanding the catalytic mechanism of sirtuins as well as the development of inhibitors and activators.

Typically, sirtuins have been characterized using either radioactive labeled cofactors [7,8] or peptide substrates [9,10] or assays employing larger peptides in combination with their analysis by HPLC [8,11], fluorescence resonance energy transfer [12] or by a mobility shift assay [13]. Furthermore, deacetylation of protein substrates such as cytochrome c and glutamate dehydrogenase was monitored by an enzyme-linked immunosorbent assay [14]. A commercial microplate reader assay is the Fluor-de-Lys assay which utilizes an acetylated tetrapeptide labeled with 7-amino-4-methylcoumarin (AMC) at the N-terminus. Upon deacetylation, the peptide becomes a substrate for trypsin releasing the fluorophore resulting in an increase of fluorescence [15,16]. A CE-based assay for sirtuin enzymes has been established employing 9-fluorenylmethoxycarbonyl (Fmoc)-labeled peptides derived from the amino acid sequence of the p53 protein, i.e. Fmoc-KK(Ac)-NH_2, Fmoc-KK(Ac)L-NH_2, and Fmoc-RHKK(Ac)-NH_2, as substrates [17,18].

Most of the assays described above require the synthesis of labeled (peptide) substrates including multistep synthesis and purification which is generally time consuming. One way to avoid these disadvantages is to monitor either the co-product nicotinamide or the co-factor NAD^+. This has been achieved by a spectroscopic assay coupling the co-product nicotinamide to $NAD(P)^+$ via a sequence of enzymatic reactions [19] or a fluorescence assay exploiting the conversion of unreacted NAD^+ to a fluorescent adduct [20]. Nevertheless such assays require a number of sequential reactions and are not trivial to perform. Another approach is the use of simpler non-peptide substrates. The general feasibility of this approach has been demonstrated in a microplate reader assay using N-benzyloxycarbonyl-lysine labeled with 7-amino-4-methylcoumarin (AMC) at the C-terminus (Z-K(Ac)-AMC, Z-MAL) as substrate [21,22]. This assay is based on the same principal as the commercial Fluor-de-Lys™ assay requiring the cleavage of the deacetylated substrate by trypsin to yield fluorescent AMC. In light of the previously developed CE assay of sirtuin enzymes [17,18] the present study was conducted in an effort to establish non-peptide substrates in a CE-based assay. Generally, CE has been established as a fast and highly efficient technique for the analysis and kinetic characterization of various enzymes [23–26].

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2. Materials and methods

2.1. Chemicals and reagents

Human recombinant SIRT1, resveratrol and Ro 31-8220 (3-[1-(3-amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide) were purchased from Enzo Life Sciences GmbH (Lörrach, Germany). NAD^+ was obtained from Sigma–Aldrich (Steinheim, Germany). Sirtinol, tris(hydroxymethyl)aminomethane (Tris), trichloroacetic acid and bovine serum albumin (BSA) were from Merck (Darmstadt, Germany). GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide) was obtained from Applichem GmbH (Darmstadt, Germany) and phosphoric acid was from Carl Roth GmbH (Karlsruhe, Germany). Protected amino acid derivatives and Rink amide-MBHA resin were from Bachem AG (Weil am Rhein, Germany). 8-Bromo-α-phenylsplitomicin and 8-bromo-β-phenylsplitomicin were a gift from Prof. Manfred Jung (University of Freiburg, Germany). The suramin analogs were a gift from Prof. Matthias Kassack (University of Düsseldorf, Germany). All other chemicals were of analytical grade. Solutions were prepared in double-distilled water.

2.2. Synthesis of substrates

Dns-K(Ac)-NH_2: 135 mg (0.5 mmol) dansyl chloride in 5 mL dichloromethane was added to 95 mg (0.5 mmol) K(Ac)-NH_2 prepared by catalytic dehydrogenation of commercial Z-K(Ac)-NH_2 (Bachem AG, Weil am Rhein, Germany) and 200 mg (2 mmol) triethylamine in 10 mL dichloromethane under stirring at 0 °C. The mixture was stirred overnight at room temperature. The organic phase was extracted three times with 10 mL water, dried over sodium sulfate and evaporated to dryness under reduced pressure to yield a greenish fluorescent solid.

Dns-K-NH_2: 140 mg (0.5 mmol) dansyl chloride in 5 mL dichloromethane was added to 140 mg (0.5 mmol) K(Boc)-NH_2·HCl (Bachem AG, Weil am Rhein, Germany) and 200 mg (2 mmol) triethylamine in 10 mL dichloromethane under stirring at 0 °C. The mixture was stirred overnight at room temperature. The organic phase was extracted three times with 10 mL water, dried over sodium sulfate and evaporated to dryness under reduced pressure. The resulting solid was treated at room temperature with 6 M HCl in dioxane for 3 h. Evaporation at reduced pressure gave a greenish fluorescent solid.

DMAC-K(Ac)-NH_2: 0.4 g Rink-amide MBHA (4-methylbenzhydrylamine, 0.56 mmol/g) resin was deprotected with 20% piperidine in DMF, followed by washing with DMF and dichloromethane. Subsequently, Fmoc-K(Ac)-OH (4 equiv.), HBTU (O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate, 4 equiv.), HOBt (hydroxybenzotriazole, 4 equiv.) and DIEA (N,N-diisopropylethylamine, 8 equiv.) in DMF were added to the resin. The reaction was allowed to proceed for 1 h after which the coupling step was repeated for 30 min following a washing step with DMF in between. Upon washing with DMF the Fmoc group was removed by treatment with 20% piperidine in DMF. After washing with DMF and dichloromethane, 7-dimethylaminocoumarin-4-acetic acid [27] was attached using the same chemistry as described for the amino acid. The product was cleaved from the resin with 95% TFA, 2.5% TIPS (triisopropylsilane) and 2.5% water for 3 h at room temperature. After precipitation in cold diethyl ether, peptides were centrifuged and washed several times with diethyl ether prior to lyophilization from water and 80% tert-butanol, respectively, to yield a yellow solid.

DMAC-K-NH_2: the compound was prepared as described for DMAC-K(Ac)-NH_2 using Fmoc-K(Boc)-OH. Simultaneous deprotection of the ε-amino group was achieved during the acid-mediated cleavage of the product from the resin. The product was obtained as a yellow solid.

All compounds were characterized by MALDI-TOF-MS. The purity exceeded 95% as determined by CE.

2.3. Capillary electrophoresis

CE experiments were performed on a Beckman P/ACE 5510 Instrument (Beckman Coulter, Krefeld, Germany) equipped with a diode array detector. 50 μm I.D. fused-silica capillaries with an effective length of 30 cm and a total length of 37 cm were from Polymicro Technologies (Phoenix, AZ, USA). New capillaries were rinsed with 1.0 M aqueous sodium hydroxide for 30 min, water for 5 min, 1.0 M hydrochloric acid for 30 min, and water for 5 min. Corrected peak areas were used for analyte quantification.

For Dns-K(Ac)-NH_2 as substrate the BGE consisted of a 200 mM Tris-phosphate buffer, pH 2.7. Separations were performed at 20 kV and 25 °C. UV detection at 230 nm was performed at the cathodic end. Samples were introduced by electrokinetic injection for 60 s at 5 kV. Between analyses the capillary was rinsed with the background electrolyte for 3 min.

The MEKC assay using DMAC-K(Ac)-NH_2 as substrate utilized a BGE consisting of a 50 mM of Tris buffer, pH 9.1, containing 180 mM SDS. Separations were carried out at 20 kV and 25 °C. UV detection at 256 nm was performed at the cathodic end. Samples were introduced by hydrodynamic injection for 95 s at 0.5 psi. Between analyses the capillary was rinsed with 0.1 M sodium hydroxide for 2 min, water for 2 min and the background electrolyte for 3 min.

2.4. SIRT1 assay

10 mM stock solutions of the substrates were prepared in H_2O containing 30% DMSO and stored at −30 °C. 10 mM NAD^+ stock solutions were prepared in the assay buffer immediately before use. Incubations were performed in a 50 mM Tris buffer, pH 8.0, containing 137 mM sodium chloride, 2.7 mM potassium chloride, 1 mM magnesium chloride and 1 mg/mL BSA. The enzyme activity was 0.1 U/μL (1.67×10^{-9} kat/μL) for Dns-K(Ac)-NH_2 and 0.05 U/μL (8.35×10^{-10} kat/μL) in the case of DMAC-K(Ac)-NH_2. The concentration of NAD^+ was 500 μM. Prior to the assay, enzyme and substrate solutions were preincubated at 37 °C for 10 min. The reaction was initiated by mixing enzyme and substrate solutions and carried out at 37 °C. In case of the Dns-K(Ac)-NH_2 CZE assay 5 μl aliquots were withdrawn and added to 20 μL of ice-cold acetonitrile containing 1% (v/v) trichloroacetic acid (TCA) kept on ice for 15 min and centrifuged at 10,000 rpm for 10 min. In case of the DMAC-K(Ac)-NH_2 MEKC assay 10 μL aliquots were withdrawn and added to 10 μL ice-cold 2.5% aqueous TCA and centrifuged at 10,000 rpm for 10 min. The supernatants were introduced into the capillary. Blank and control experiments were performed by either omitting SIRT1 or NAD^+ in the incubation mixtures.

For the determination of the enzyme kinetic parameters, K_m, V_{max}, k_{cat} and k_{cat}/K_m, concentrations of the substrates ranged between 0 and 300 μM. An incubation time of 8 min was selected to calculate the initial velocity of deacetylation. Each concentration was determined in three independent experiments. The plots of initial velocities versus substrate concentration were fitted to the Michaelis–Menten equation.

2.5. Evaluation of activators and inhibitors

The potential activation of SIRT1 by resveratrol as well as inhibition studies were carried out as described above for the general assay. The concentration of resveratrol was 200 μM. The inhibition of SIRT1 was characterized by the determination of the remain-

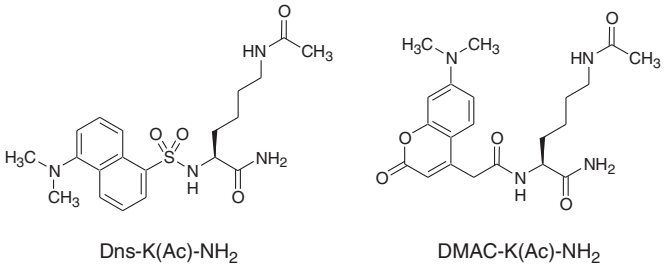

Fig. 1. Structures of the substrates Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2.

ing activity of the enzyme at inhibitor concentrations of 50 μM. DMSO was added at concentrations <5% (v/v) to ensure dissolution of the inhibitors in the incubation mixture. Control experiments contained DMSO at the same concentration. Each experiment was performed in triplicate, each sample was analyzed twice.

3. Results and discussion

3.1. Synthesis of substrates

Small peptides containing a fluorescence label at the N-terminus have been successfully established as substrates for sirtuin enzymes in CE-based assays [17,18]. The question was if a further reduction of the substrate structure to a single amino acid would be feasible. Thus, lysine amide containing a fluorescence label at the nitrogen was selected as template. The structures of the substrates Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2 are shown in Fig. 1. Both compounds are based on the amino acid lysine. The amide function was selected in order to provide some similarity to peptide structures while the amino group was derivatized with the Dns and DMAC groups to provide a chromophor. Both substrates as well as the corresponding deacetylated products were synthesized by standard solution or solid phase procedures. The identity of the compounds was confirmed by MALDI TOF mass spectrometry.

3.2. Capillary electrophoresis method development

The initial assay conditions were based on the previously developed method employing Fmoc-labeled small peptide substrates [17,18]. The enzyme reaction was quenched by the addition of TCA and CZE analysis was performed using a 200 mM Tris-phosphate buffer, pH 2.7. However, under these conditions severe peak deformation for Dns-K(Ac)-NH_2 and the product Dns-K-NH_2 as well as peaks splitting in the case of the co-product nicotinamide was observed due to the low pH and high salt content of the injected sample (Fig. 2A). Sodium hydroxide-mediated sample stacking as applied for the peptide substrates was ineffective probably due to the low basicity of the Dns group. Therefore, field-amplified sample injection (FASI) from a solution containing a high ACN content was evaluated. Thus, the enzyme reaction was quenched using a 4-fold excess of ice-cold ACN containing TCA. The high ACN content led to protein denaturation while TCA protonated the analytes to enable FASI. The TCA concentration was optimized in the range of 0.2–5% (v/v). FASI was performed at 5 kV. A concentration of 1% (v/v) was found to be sufficient for effective protonation of the analytes while at the same time avoiding a large increase in conductivity of the sample which would impair the stacking effect of FASI. Subsequently, injection times between 10 s and 75 s were investigated and 60 s was selected to give adequate assay sensitivity and peak shape (Fig. 2B). Overall application of FASI led to a moderate increase in sensitivity by a factor of 3 in the case of Dns-K(Ac)-NH_2, by a factor of 5 for Dns-K-NH_2 and by a factor of 2 for nicotinamide compared to the initial conditions. An advantage of the dilution

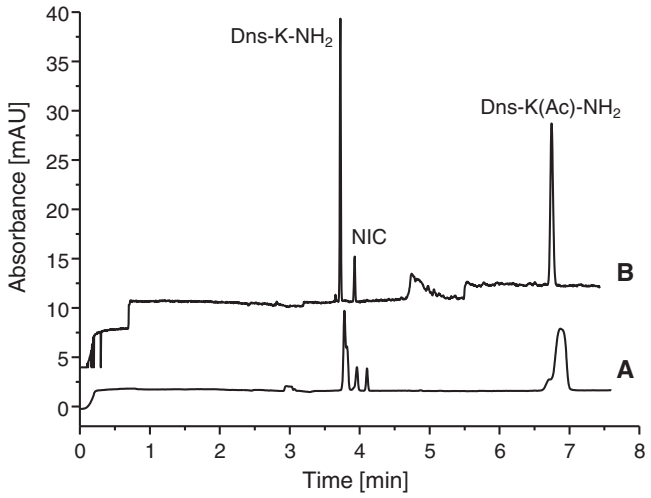

Fig. 2. CZE separation of Dns-K(Ac)-NH_2, Dns-K-NH_2 and nicotinamide following (A) hydrodynamic sample injection for 5 s and (B) FASI at 5 kV for 60 s. Experimental conditions: 37/30 cm, 50 μm I.D. fused silica capillary; 200 mM Tris-phosphate buffer, pH 2.7, 20 kV, 25 °C, 230 nm. Sample: Dns-K(Ac)-NH_2, Dns-K-NH_2 and nicotinamide dissolved in enzyme assay buffer at 100 μM, (A) addition of 2.5% (v/v) TCA for direct injection, (B) addition of 4 volumes ACN containing 1% (v/v) TCA. NIC, nicotinamide.

of the sample by ACN is the use of small aliquots of the incubation mixture resulting in a reduction of the use of enzyme and substrate.

Using a pH 2.7 BGE in the CZE mode DMAC-K(Ac)-NH_2 could not be detected within 10–15 min (Fig. 3A) due to the low basicity of the 7-dimethylaminocoumarin moiety. The compound eluted around 25 min (data not shown). Long migration times are not desirable for an effective enzyme assay. Upon lowering the pH of the BGE to 1.8 the compound migrated at about 12 min but sensitivity was low due to high baseline noise. Therefore, a MEKC method was developed for the determination of DMAC-K(Ac)-NH_2 and the deacetylated product DMAC-K-NH_2. A 50 mM Tris buffer, pH 9.1, containing SDS allowed the analysis of both compounds within 12 min. In order to increase assay sensitivity a sweeping technique was applied. In

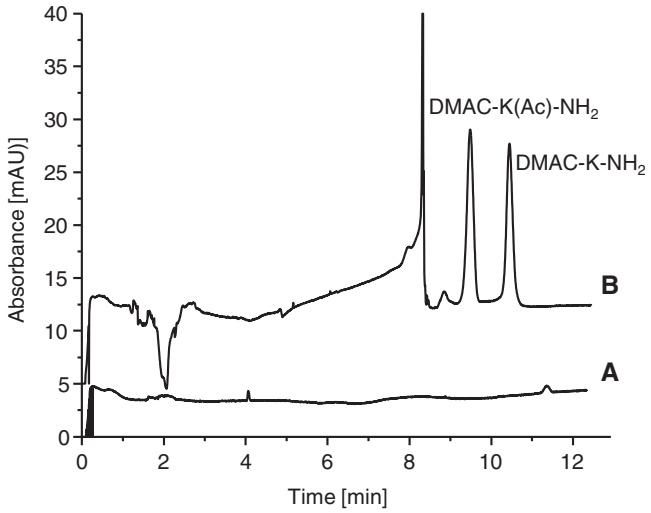

Fig. 3. CE analysis of DMAC-K(Ac)-NH_2 and DMAC-K-NH_2. (A) CZE in 200 mM Tris-phosphate buffer, pH 2.7, hydrodynamic sample injection for 5 s. (B) MEKC in 50 mM Tris buffer, pH 9.1 containing 180 mM SDS, hydrodynamic sample injection for 95 s. Other experimental conditions: 37/30 cm, 50 μm I.D. fused silica capillary, 20 kV, 25 °C, 256 nm. Sample: analyte concentration 50 μM in enzyme assay buffer, (A) addition of 2.5% (v/v) TCA for direct injection, (B) addition of 1 volume 2.5% aqueous TCA.

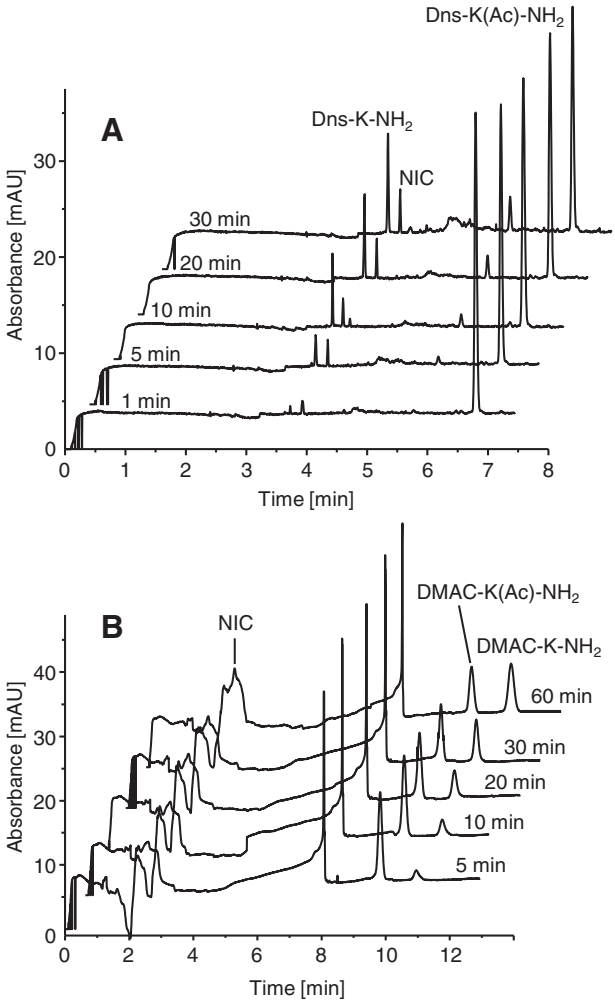

Fig. 4. Time course the deacetylation of (A) Dns-K(Ac)-NH_2 and (B) DMAC-K(Ac)-NH_2 by SIRT1. Experimental conditions: 37/30 cm, 50 μm I.D. fused silica capillary, 25 kV, 25 °C; (A) 200 mM Tris-phosphate buffer, pH 2.7, FASI at 5 kV for 60 s, 230 nm; (B) 50 mM Tris buffer, pH 9.1 containing 180 mM SDS, hydrodynamic sample injection for 95 s, 256 nm. NIC, nicotinamide.

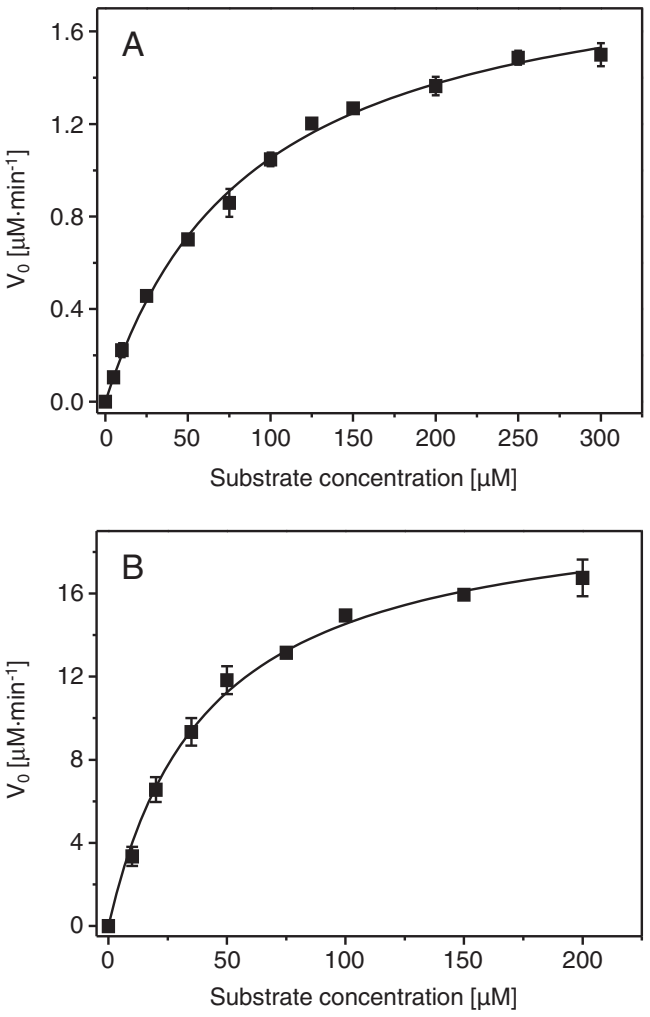

Fig. 5. Enzyme kinetic data of SIRT1 using (A) Dns-K(Ac)-NH_2 and (B) DMAC-K(Ac)-NH_2 as substrates. Each data point is the mean ± SD ($n = 3$). Enzyme activity 0.1 U/μL for Dns-K(Ac)-NH_2 and 0.05 U/μL for DMAC-K(Ac)-NH_2, for further experimental details see text.

order to achieve similar conductivity of sample and BGE and to quench the enzyme reaction as well, 2.5% aqueous TCA was added to the incubation mixture at a ratio of 1:1. The concentration of SDS was varied between 50 and 180 mM. The highest effect on analyte concentration by sweeping was achieved at 180 mM SDS which was subsequently applied. Optimization of the injection time led to a time of 95 s. An electropherogram obtained with the optimized MEKC conditions is shown in Fig. 3B. Compared to a simple injection for 5 s sensitivity increased by a factor of 24 for DMAC-K-NH_2 and by a factor of 14 for DMAC-K(Ac)-NH_2 upon application of the sweeping technique. The peaks at about 2.3 min and 8 min are system peaks caused by the addition of TCA for the quenching process.

A disadvantage of the MEKC method is the fact that nicotinamide cannot be detected due to poor concentration during the sweeping as well as its comigration with sample matrix components at about 2.3 min.

3.3. Method validation

Both optimized methods were validated with respect to range, linearity, LOQ, LOD and recovery. Range was examined using six concentration levels. LOQ was estimated at a signal-to-noise ratio of 10 while LOD corresponded to a signal-to-noise ratio of 3. The recovery was estimated by comparison of standards dissolved in assay buffer without BSA compared to standards dissolved in assay buffer containing BSA followed by protein precipitation and centrifugation. Assay precision with regard to migration time and corrected peak area was determined at the 50 μM level in the case of Dns-K(Ac)-NH_2, Dns-K-NH_2 and nicotinamide and at the 100 μM level for DMAC-K(Ac)-NH_2 and DMAC-K-NH_2. For intraday precision the samples were injected six times on the same day while interday precision was assessed analyzing each sample in triplicate on three consecutive days. The data are listed in Table 1. Generally both assays are comparable based on the performance data. Repeatability of migration time appeared to be slightly better in the CZE assay compared to the MEKC mode. As stated above, nicotinamide could not be analyzed with the MEKC method due to interference with sample components.

3.4. Sirtuin assay

The deacetylation of the substrates Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2 by human recombinant SIRT1 was monitored with both CE methods. Both substrates were effectively metabolized by SIRT1 as shown in Fig. 4 using a concentration of the substrates of 100 μM. After incubations times of 1–5 min the respective deacetylated products could be clearly identified. In the absence of the cofac-

Table 1
Calibration data of CE enzyme assays.

	CZE assay			MEKC assay	
	Dns-K(Ac)-NH_2	Dns-K-NH_2	Nicotinamide	DMAC-K(Ac)-NH_2	DMAC-K-NH_2
Linearity (μM)	4.0–100	2.5–100	12.5–200	5.0–150	7.0–150
Coefficient of determination (r^2)	0.996	0.997	0.998	0.999	0.997
LOD (μM)	1.2	0.7	3.8	1.6	2.4
LOQ (μM)	4.0	2.5	12.5	4.7	7.0
Recovery (%)	99.8 ± 4.3	98.1 ± 1.8	101.0 ± 3.9	93.3 ± 2.8	101.0 ± 4.1
Intraday precision[a]					
Migration time (RSD %)	0.5	0.9	0.7	1.7	1.8
Peak area (RSD %)	3.2	4.5	3.9	4.3	2.5
Interday precision[a]					
Migration time (RSD %)	0.7	1.0	1.1	2.1	2.7
Peak area (RSD %)	3.8	4.5	6.9	7.7	5.4

[a] Determined at 50 μM for Dns-K(Ac)-NH_2, Dns-K-NH_2 and nicotinamide and at 100 μM for DMAC-K(Ac)-NH_2 and DMAC-K-NH_2.

tor NAD^+ or the enzyme substrate conversion could not be observed (data not shown) clearly indicating that cleavage of the acetyl group was mediated by SIRT1. In incubations using Dns-K(Ac)-NH_2 as substrate an additional peak appeared at about 5.7 min which also increased with time. There is no explanation for the peak at this time but it is subject to further investigations.

3.5. Enzyme kinetics

For further substrate characterization Michaelis–Menten kinetics were determined for Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2. Experiments were performed using SIRT1 activities of 0.1 U/μL for Dns-K(Ac)-NH_2 and 0.05 U/μL for DMAC-K(Ac)-NH_2 while concentrations of the substrates varied between 0 and 300 μM. Fig. 5 shows the dependence of the deacetylation initial velocity, v_0, on the substrate concentration. The kinetic data, i.e. the Michaelis constant, K_m, the maximum velocity, V_{max}, the turnover number, k_{cat} and the specificity constant, k_{cat}/K_m, are summarized in Table 2.

The K_m values of 88.0 μM and 42.9 μM obtained for Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2, respectively, are about 2.5- to 5-fold higher compared to the values achieved previously for Fmoc-labeled small peptide substrates which ranged between 16.7 and 34.6 μM [18]. However, the constants are within the same order of magnitude reported for other sirtuin peptide substrates ranging between 11.9 and 108 μM [7,13,28,29]. SIRT1 has a higher affinity towards DMAC-K(Ac)-NH_2 than Dns-K(Ac)-NH_2 and deacetylated DMAC-K(Ac)-NH_2 at an approximately 20-fold higher rate compared to Dns-K(Ac)-NH_2. The catalytic efficiency represented by the k_{cat}/K_m value was also higher in the case of DMAC-K(Ac)-NH_2. The value is in the range of values reported for other peptide substrates in the literature [7,30].

Table 2
Kinetic parameters for SIRT1 deacetylation of Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2. The values are the mean ± SD (n = 3).

	Dns-K(Ac)-NH_2	DMAC-K(Ac)-NH_2
K_m (μM)	88.0 ± 2.0	42.9 ± 1.9
V_{max} (μM min^{-1})	2.0 ± 0.1	20.7 ± 0.4
k_{cat} (s^{-1})	0.083 ± 0.003	1.75 ± 0.04
k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	0.92 ± 0.01	39.6 ± 0.9

3.6. Effect of resveratrol on substrate deacetylation by SIRT1

The report of the activation of SIRT1 by resveratrol, a naturally occurring polyphenol has attracted attention in the past, decreasing the K_m values of both, the acetylated substrate and NAD^+ [9,15]. However, as pointed out in recent studies the activation appears to be an artifact that is only observed when small peptide substrates labeled with AMC at the C-terminus are employed as substrates [31,32]. SIRT1 activation by resveratrol was not observed when the natural substrates such as p53, acetylated PGC-1α or acetyl-CoA synthetase1 were studied. In the present study, resveratrol at concentrations of 200 μM did not increase the SIRT1-mediated deacetylation of Dns-K(Ac)-NH_2 or DMAC-K(Ac)-NH_2 indicating that the non-peptide compounds resemble the natural substrates in this respect. As stated above, activation by resveratrol was observed when the C-terminus of a small peptide is labeled with AMC. DMAC-K(Ac)-NH_2 contains a coumarin residue at the "N-terminus" of lysine. No activation could be demonstrated in the case of this substrate. Thus, it may be speculated that a coumarin residue alone is

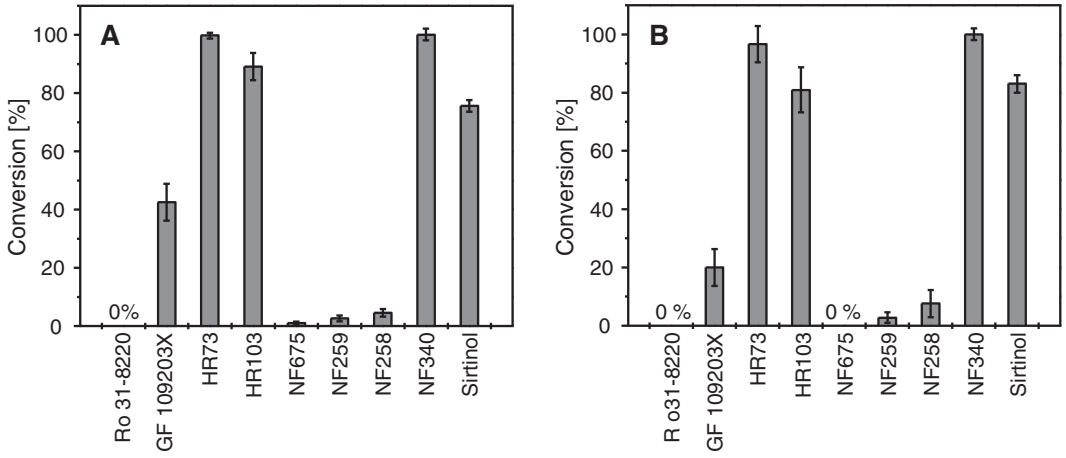

Fig. 6. Inhibition of SIRT by inhibitors using (A) Dns-K(Ac)-NH_2 and (B) DMAC-K(Ac)-NH_2 as substrates. The data are the mean ± SD (n = 3). For experimental details see text.

not sufficient for the activation phenomenon but the group has to be in a certain position with regard to the acetyl-lysine residue in a peptide.

3.7. Inhibitor screening

Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2 were further evaluated for their use in the determination of sirtuin inhibitors. Thus, known inhibitors were coincubated with the substrates. The inhibitors included sirtinol [33], two bisindolylmaleimides (Ro 31-8220 and GF 109203X) [34], two splitomicin derivatives (HR73 and HR103) [22] and four suramin analogues (NF675, NF258, NF259 and NF 340) [35]. Fig. 6 summarizes the deacetylation of the substrates at a concentration of 100 μM in the presence of 50 μM inhibitor by SIRT1 after an incubation time of 8 min. Essentially identical results were obtained for both substrates in the two assays. Ro 31-8220 and the suramin analogs NF675, NF258 and NF259 proved to be the most effective inhibitors reducing the conversion of both substrates below 10%. The order of the suramin inhibitors was comparable to reported data [35]. Low inhibitory activity of the splitomycin derivatives, HR73 and HR103, has been previously observed using Fmoc-labeled peptides [18]. This has been attributed to the low aqueous solubility of the compounds. In contrast to the assay employing Fmoc-labeled peptides [18], sirtinol proved to be a poor inhibitor of SIRT1 with the non-peptide substrates. DMSO at concentrations up to 5% did not affect the activity of the enzyme.

4. Conclusions

Two non-peptide substrates for SIRT1, Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2, have been synthesized by directly modifying acetyl-lysine amide at the α-amino group with a dansyl or 7-dimethylaminocoumarin residue. Two CE assays including suitable preconcentration techniques, i.e. a CZE method with FASI and a MEKC method combined with analyte sweeping, have been developed and validated. Both assays proved to be suitable to monitor the deacetylation process of the substrates and the possibility of screening the activity of enzyme inhibitors was demonstrated.

Enzyme kinetic data were essentially comparable to reported values of larger substrates [13,18,28–30], implying the applicability of small non-peptide substrates for sirtuin assay. It is important to note that no activation by resveratrol was observed for the non-peptide substrates as it is the case for native sirtuin substrates. Differences in the catalytic efficiency of SIRT1 towards Dns-K(Ac)-NH_2 and DMAC-K(Ac)-NH_2 indicate that the chromophor bound to the α-amino group of lysine affects the affinity of the enzyme toward the substrate and/or the reaction. Nevertheless, the results illustrate a "structural tolerance" of SIRT1 with regard to the substrate. This includes the exchange of an amide (peptide) bond by a sulfonamide as found in Dns-K(Ac)-NH_2.

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