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ABSTRACT

Objective We attempted to overexpress Human Histone Deacetylase 1 (HDAC1) in *Escherichia coli*.

Results A synthetic gene coding for HDAC1, and optimised for *E. coli* codon usage, was cloned into pBADHisB, generating pBAD-rHDAC1. This construct was used to transform *E. coli* TOP10, and the target protein was overexpressed and partially purified. According to its elution volume from a Superdex 200 column, the partially purified rHDAC1 was obtained in aggregated form, i.e. as an octamer. The dissociation of octameric HDAC1 was tested using several agents, among which sodium dodecyl sulfate was competent in partially dissociating rHDAC1 aggregates. When the enzyme activity was tested *in vitro* using ^{3}H-acetyl-labelled histones both protein samples, aggregated and dissociated, were active. Hence, our results suggest that *E. coli* represents an alternative system for the production of the recombinant HDAC1.

Conclusions We described a procedure for the overexpression in *E. coli* of recombinant HDAC1, the purification of which in active form can be successfully performed, although yielding an octameric aggregate.

Key words: *Escherichia coli*; heterologous expression; human histone deacetylase 1 (HDAC1); protein dissociation; protein purification.

INTRODUCTION

Histone deacetylases (HDACs) are specifically responsible for the deacetylation of lysine residues at the N-terminal regions of the core histones (H2A, H2B, H3 and H4). In addition, HDACs may deacetylate other non-histone proteins and thus are involved in several cellular processes (e.g. differentiation, apoptosis, cancer development).

To date, human histone deacetylases belonging to all three known classes (class I: HDAC1, HDAC2, HDAC3, HDAC8; class II: HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10; class III: HDAC11) have been produced using the baculovirus-insect cell expression system. However, all these recombinant proteins were generally expressed as GST/His/FLAG-tagged proteins with limited yield and high costs. Alternatively, the heterologous expression system based on *Escherichia coli* provides several advantages, such as the simple and well-known genetic manipulation, the high cell density achievable, the short time for cultivation and the cheapness of substrates. Despite these advantages, the use of *E. coli* as an alternative expression system for HDACs has been limited. Recently, the full length HDAC8, belonging to the class I, was produced using *E. coli* BL21(DE3) cells. In particular, the protein was fused either to GST or His-tag at the N-terminal and the overexpression was triggered by the addition of IPTG to the culture medium (Feng et al. 2011). Both recombinant proteins, mostly recovered in the insoluble fraction, were active after purification using GST or Ni-NTA resin columns, respectively, followed by Source Q and Superdex-200 columns.

The expression of His-tagged HDAC1 in HEK293 cells transformed with the pcDNA3.1 vector was attempted (Li et al. 2004). In this case, the yield of recombinant protein was very low, albeit the recovered enzyme was shown to be active. To investigate the role of *in vivo* and *in vitro* phosphorylation at S421 and S423 sites, the gene coding for HDAC1 was recently cloned into the pET21b vector and the enzyme was overexpressed as His-tagged protein in *E. coli* BL21(DE3) (Karwowska-Desaulniers et al. 2007). However, the production of HDAC1 with high yields has not been described yet. Nevertheless, recombinant HDAC1 is commercially available, although with low

purity and at high cost. To improve the efficiency of recombinant HDAC1 production, we report here on a procedure for the production of this enzyme devoid of any tag, using *E. coli* as the expression host.

MATERIALS AND METHODS

Bacterial strain, plasmid and media

Escherichia coli TOP10 was used for the expression of recombinant HDAC1 (rHDAC1). A synthetic gene, optimised for *E. coli* codon usage using the software Leto® (Entelechon GmbH, Germany), was cloned into the pBADHisB expression vector (Invitrogen, USA), using the *Nco*I and *Pst*I restriction sites, yielding the recombinant pBAD-rHDAC1 plasmid. *E. coli* TOP10 competent cells were transformed by electroporation with 20 ng of the recombinant construct, and transformants were selected on LB agar plates containing ampicillin (100 μg/mL). Bacterial cultures were grown in LB medium (10, 5, and 10 g/L of tryptone, yeast extract, and NaCl, respectively) supplemented with ampicillin (100 μg/mL).

Optimization of protein expression

Single colonies of *E. coli* TOP10/pBAD-rHDAC1 were grown overnight at 37 °C in 5 mL of LB/ampicillin. The pre-cultures accordingly obtained were diluted (1:250) in 20 mL of fresh medium, and grown for 8 h at 37 °C, under constant shaking (200 rpm). The expression of the target protein was tested as a function of temperature (30 or 37 °C), of the concentration of arabinose (1.3 or 13 mM) and of induction time (2.5, 6 and 15 h). To analyse the expression of rHDAC1, cells pellets were resuspended in 10% SDS (m/v), 10 mM β-mercaptoethanol, 30% glycerol (v/v), 0.2 M Tris-HCl pH 6.8, 0.05% bromophenol blue (m/v) and boiled for 5 min. 18 μL of each sample were subjected to SDS-PAGE in 10% polyacrylamide gels.

rHDAC1 production

After growing a culture at 37 °C for 8 h, the expression of rHDAC1 was induced with 1.3 mM arabinose at 30 °C for 15 h. The cells suspension (3 L) was centrifuged and the pellets were resuspended in 40 mL of buffer A (50 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA, 2.5 mM DTT) supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF). Cells were homogenized using a cold potter, then sonicated on ice (7 cycles, 15 sec pulses with 15 sec intervals, at 18 W for 2 min). Soluble proteins were recovered by centrifugation at 10,000xg for 30 min. Protein concentration was then determined according to Bradford (Bradford 1976).

rHDAC1 purification

After filtration, the protein extract (40 mL) was loaded onto a Q-Sepharose FF column (1.6 x 25 cm, 50 mL volume) equilibrated with buffer A. Following a washing step with the same buffer, the elution was performed by a linear 50-600 mM NaCl gradient. Eluted fractions, diluted 1:2 with buffer B (buffer A containing 40% glycerol), were subjected to SDS-PAGE analysis. The best fractions were pooled, diluted in buffer C (50 mM Tris-HCl pH 8, 1 mM EDTA, 20% glycerol) and concentrated by ultrafiltration. The resulting sample was loaded onto a HiTrap Heparin affinity column (5 mL, GE Healthcare, USA) equilibrated with buffer D (50 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA, 20% glycerol). After washing, a linear 50-800 mM NaCl gradient was applied and fractions of 0.8 mL were collected. The flow-through was concentrated and then applied to a poly-L-lysine agarose (Sigma-Aldrich, USA) affinity column (1.5 x 5 cm, 8 mL volume) previously equilibrated with buffer D. The elution was performed with a linear gradient from 50 mM to 1 M NaCl and the presence of rHDAC1 was evaluated by SDS-PAGE. The final purification step was performed with a Superdex 200 column (1.6 x 70 cm), previously calibrated with Broad-Molecular-Weight protein standards (GE Healthcare, USA), and equilibrated with 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 2.5 mm DTT.

Mass spectrometry

Elution of proteins from acrylamide gels, trypsin digestion, and separation of peptides were performed as previously described (Shevchenko et al. 2007; Conte et al. 2012).

Dissociating agents

In order to obtain rHDAC1 in monomeric form, different conditions and dissociating agents were tested: aliquots of purified rHDAC1 (2.5 μg) were incubated at room temperature for 1 h in the presence of each agent. The samples were conditioned with native sample buffer (40% v/v glycerol, 0.4 M Tris-HCl pH 6.8, 0.05% bromophenol blue), then loaded on native polyacrylamide gels (7.5 and 4% acrylamide for running and stacking gel, respectively). Additional samples, prepared by mixing 5 μg of rHDAC1 with each dissociating agent in a volume of 100 μL, were loaded onto Amicon Ultracel 100,000 NMWL filters (Millipore, USA), and centrifuged for 45 s at 14,000xg. Both eluate and retentate fractions were subjected to SDS-PAGE analysis (10% acrylamide).

Preparation of [^{3}H]acetyl histones and assay of HDAC1 activity

To obtain [^{3}H]acetyl-labelled histones as the substrate for the HDAC1 assay, 1x10^7 HT29 cells were incubated in 30 mL of medium containing 0.5 mCi/mL [^{3}H]acetate for 1 h, and the labeled histone fraction was immediately extracted. Cells were harvested using 0.11% trypsin and 0.02% EDTA, washed twice with 10 mM sodium butyrate in PBS, and nuclei were isolated (Amellem et al. 1996). Sodium butyrate (5 mM) was added to nuclear isolation buffer to prevent histone deacetylation. The nuclear pellet was suspended in 0.1 mL of ice-cold water using a Vortex mixer, and concentrated H_2SO_4 was added to the suspension to give a final concentration of 0.4 M. After incubation at 4 °C for 1 h, the suspension was centrifuged for 5 min at 14,000xg, and the supernatant was taken and mixed with 1 mL of acetone. After overnight incubation at -20 °C, the coagulate material was collected by microcentrifugation and air-dried. This acid-soluble histone fraction was dissolved in 50 μl of water. Protein concentration was determined by the Bradford assay (Bradford 1976).

HDAC1 activity was estimated in 50 μl of a reaction mixture containing 1.5 or 2.5 μg HDAC1, [^{3}H] acetate-labeled HT29 histones (12,000 dpm) dissolved in HDA buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol) at 37 °C for 2 h. The reaction was stopped by the addition of 50 μl of 1 M HCl and 0.16 M acetic acid. The released [^{3}H]acetic acid was extracted with 0.5 mL of ethyl acetate,

and the solvent layer was taken into 5 mL of toluene scintillation solution for the determination of radioactivity.

RESULTS AND DISCUSSION

Expression in *E. coli*

We attempted the heterologous expression of the human histone deacetylase 1 (HDAC1) in a prokaryotic system. The full-length human HDAC1, GST/His or FLAG-tagged, was produced in baculovirus infected insect cells (Hassig et al. 1997; Hassig et al. 1998). Recently, the expression of the whole human HDAC1 protein in mammalian cells has been achieved (Li et al. 2004). However, in this study, the recombinant HDAC1 was obtained as His-tagged protein (at the N-terminal). So far, several attempts have been made to use microbial expression hosts, like *Pichia pastoris* and *Escherichia coli*, but all the systems tested were unsuccessful. The only mammalian histone deacetylase expressed in *E. coli* was HDAC8 (Feng et al. 2011; Hu et al. 2000): the coding sequence, cloned into pET21b plasmid, was expressed as His-tagged protein using BL21(DE3) cells after induction with IPTG. The recombinant HDAC8-his protein, after purification by nickel affinity column, was found to be active despite the absence of post-translational modifications.

In view of these considerations, we have attempted to express recombinant HDAC1 in *E. coli*. To this aim, the synthetic gene, optimised for *E. coli* codon usage (Supplementary Fig. 1), was cloned into the tightly regulated vector, pBADHisB, which contains the *ara*BAD promoter (Guzman et al. 1995). In order to obtain rHDAC1 without any additional sequence, the cloning was performed using proper restriction sites (*Nco*I and *Pst*I) to eliminate the N-terminal histidine tag eventually conferred by the vector.

Preliminary experiments were performed in small shake-flasks to test different conditions of growth and induction (e.g. time/temperature of growth and inducer concentration) in order to identify the optimal procedure for the heterologous expression. In particular, we tested two different inducer

concentrations, 1.3 and 13 mM, and two temperatures of induction, 30 and 37 °C. As shown in Fig. 1A (lane 1 and 2), the band corresponding to rHDAC1 was detectable, albeit weak, on denaturing gels, independently of the growth temperature. A similar independence was observed using 1.3 or 13 mM arabinose as inducer (data not shown). To prevent the possible production of protein aggregates (e.g. inclusion bodies) and to avoid cellular stress in response to the induction, we decided to induce the overexpression of HDAC1 at 30 °C with 1.3 mM arabinose, when bacterial populations reached an OD_{600} equal to 0.8-0.9. After 15 h induction at 30 °C, cells pellets were harvested and used for protein extraction.

FIGURE 1

We determined the growth kinetics of *E. coli* TOP10 populations containing the pBAD-rHDAC1 or the pBAD vector, under non-inducing or inducing conditions. The addition of arabinose (5 h after the inoculum) to induce the expression of rHDAC1 did adversely affect the biomass yield of TOP10/pBAD-rHDAC1 (Fig. 1B), most likely due to a weak toxic effect of the recombinant protein.

Purification of rHDAC1

We used four chromatographic steps to purify rHDAC1 (see Methods and Supplementary Fig.s 2-4). Starting from about 10 g of wet biomass the final recovery was equal to 2.5 mg of purified rHDAC1. Unfortunately, rHDAC1 was obtained in aggregated form, as an octamer. According to the elution volume of the protein from the gel filtration column (Fig. 2A), corresponding to a K_{av} of 0.155, the molecular mass of the recombinant protein was estimated equal to 470 kDa, whereas the expected mass is 55.1 kDa. The gel filtration peak containing fractions 8-15 (Fig. 2A) did contain HDAC1 as was confirmed by SDS-PAGE (Fig. 2B), and by mass spectrometry (Table 1).

TABLE 1, FIGURE 2

Although in aggregated form, the recombinant protein was very stable and has not been shown to be susceptible to proteolysis. In particular, 5 μg of purified rHDAC1 were incubated at 4 °C and 37 °C for 3 h, then samples were analysed by SDS-PAGE. As shown in Fig. 2C, only a single band

corresponding to rHDAC1 is visible, indicating the absence of degradation and high stability of the protein.

Protein dissociation

As previously mentioned, gel filtration chromatography showed that rHDAC1 was produced as a high molecular mass aggregate. Before testing its activity, we tried to dissociate this protein aggregate using several dissociating agents. In particular, the effect of chaotropic species, weak kosmotropes, amino acids, reducing or denaturing agents, detergents, alcohols, and saturated fatty acids was tested (Table 2).

TABLE 2

Preliminary information about the aggregation state of purified rHDAC1 was obtained by native electrophoresis, performed in the absence of denaturing or reducing agents. Under these conditions, high molecular mass complexes are inhibited from entering the gel. As shown in Fig. 3A, rHDAC1 was able to migrate further in native gels only after pre-treatment with 2% (w/v) SDS, while the incubation with several other agents was ineffective.

FIGURE 3

A detailed screening of dissociating agents was then performed with a simple and rapid assay: an aliquot of purified rHDAC1 was incubated with each agent at room temperature for 1 h, then samples were subjected to ultrafiltration using disposable cells with a molecular weight cut-off (100 kDa) able to discriminate between monomeric (55 kDa) or aggregated proteins (> dimeric, 110 kDa). Samples (100 μL) were centrifuged for 45 s at 14,000xg, then aliquots of the protein retained over the membrane (aggregates) and protein passed through the ultrafilter (monomeric form) were analysed by SDS-PAGE. Interestingly, the anionic detergent SDS was the only agent able to dissociate rHDAC1 aggregates to monomers (lane 4, Fig. 3B), in agreement with the observations obtained with native polyacrylamide gels. Unfortunately, the effect of SDS was incomplete and a significant amount of aggregated protein was still detectable in the retentate fraction.

We also analysed different SDS concentrations (ranging from 0.002 to 2% m/v, corresponding to concentrations from 0.069 to 69 mM) in order to determine the smallest able to partially dissociate rHDAC1 complexes. This dissociative effect was evident down to concentrations equal to 0.05% (corresponding to 1.75 mM, Fig. 3C), while lower concentrations did not trigger dissociation (data not shown). These observations are in excellent agreement with the dissociation action exerted by SDS (tested at concentrations from 0.13 to 10 mM) towards carmin (Rao and Prakash 1993). The minimal SDS concentration able to dissociate carmin was indeed evaluated as equal to 1.6 mM (Rao and Prakash 1993), a value very close to that reported here as effective in rHDAC1 dissociation, i.e. 1.75 mM.

Activity assays

To assess rHDAC1 activity, the aggregated enzyme was assayed using [^{3}H]acetyl histones as substrate. The amount of [^{3}H]acetic acid released from the histones was observed to linearly depend on the rHDAC1 concentration in the reaction mixture (Fig. 4).

FIGURE 4

A small, but significant, release of [^{3}H]acetic acid was detected in the absence of rHDAC1, independently of the addition of SDS to the reaction mixture (Fig. 4). However, it should also be noted that the extent of this unspecific activity accounts for 3-5% only of the activity determined in the presence of enzyme (Fig. 4). We did also compare the activity exerted by 1.5 μg of aggregated or monomeric rHDAC1, and we observed similar substrate conversion, i.e. we detected $355*10^3$ and $275*10^3$ dpm / mg histones in the presence of octameric and monomeric enzyme, respectively.

CONCLUDING REMARKS

We reported here a procedure for the overexpression of HDAC1 in *E. coli.* The protein of interest was partially purified, although as an octamer. By appropriate screens, we identified SDS as an agent able to partially dissociate this aggregated form of recombinant HDAC1. When assayed, we detected significant deacetylase activity exerted by rHDAC1, both in aggregated and dissociated form. In

particular, the activity exerted by the aggregated enzyme (Fig. 4) is very similar to the catalytic action reported for rHDAC1 expressed in mammalian cells (Li et al. 2004). Indeed, our HDAC1 preparation and the enzyme expressed in mammalian cells were both assayed with labelled histones, and yielded comparable substrate conversions: i) we incubated, at 37 °C for 2 h, 1.5 μg of enzyme and 3.6 μg of tritiated histones (featuring 5600 cpm), and we observed, under these conditions, the conversion of 18% of substrate; ii) when 1 μg of the enzyme produced in mammalian cells was incubated at 37 °C for 1 h in the presence of labelled histones, the amount of radioactive product (acetate) was determined as equal to 18% of substrate ((Li et al. 2004). These assays suggest that the activity of our octameric rHDAC1 is slightly lower than the recombinant deacetylase isolated from mammalian cells. Accordingly, *E. coli* seems to be a suitable host for the production of recombinant HDAC1.

SUPPORTING INFORMATION

Supplementary Figure 1

Sequence of the synthetic optimised rHDAC1 gene. The *Nco*I and *Pst*I restriction sites, used for the cloning into pBADHisB, are underlined. The ATG start codon in shown in bold.

Supplementary Figure 2

(A) Purification of rHDAC1 by anion exchange chromatography (Q-sepharose FF column, 50 mL). (B) SDS-PAGE (10% polyacrylamide) of eluted fractions. Lane M: molecular mass markers (116, 66, 45, 35, 25 kDa); lane I: sample of soluble protein extract (input); FT: flow-through fractions; W: washing fractions; numbered lanes: eluted fractions.

Supplementary Figure 3

(A) HiTrap Heparin affinity chromatography of rHDAC1 (5 mL column). (B) SDS-PAGE (10% polyacrylamide) of eluted fractions. Lane M: molecular mass markers (116, 66, 45, 35, 25 kDa); lane I: sample of pooled fractions (input); FT: flow-through fractions; W: washing fractions; numbered lanes: eluted fractions.

Supplementary Figure 4

(A) Poly-lysine affinity chromatography of rHDAC1 (8 mL column). (B) SDS-PAGE (10% polyacrylamide) of eluted fractions. Lane M: molecular mass markers (116, 66, 45, 35, 25 kDa); lane I: sample of pooled fractions (input); FT: flow-through fraction; W: washing fraction; numbered lanes: eluted fractions.

REFERENCES

1. Amellem O, Stokke T, Sandvik JA, Pettersen EO (1996) The retinoblastoma gene product is reversibly dephosphorylated and bound in the nucleus in S and G2 phases during hypoxic stress. Exp Cell Res 227:106–115.

2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

3. Conte E, Vincelli G, Schaaper RM, Bressanin D, Stefan A, Dal Piaz F, Hochkoeppler A (2012) Stabilization of the *Escherichia coli* DNA polymerase III ε subunit by the θ subunit favors *in vivo* assembly of the Pol III catalytic core. Arch Biochem Biophys 523:135-143.

4. Feng JH, Jing FB, Fang H, Gu LC, Xu WF (2011) Expression, purification, and S-nitrosylation of recombinant histone deacetylase 8 in *Escherichia coli*. Biosci Trends 5:17-22.

5. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-

level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177:4121-4130.

6. Hassig CA, Fleischer TC, Billin AN, Schreiber SL, Ayer DE (1997) Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell 89:341-347.

7. Hassig CA, Tong JK, Fleischer TC, Owa T, Grable PG, Ayer DE, Schreiber SL (1998) A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc Natl Acad Sci USA 95:3519-3524.

8. Hu E, Chen Z, Fredrickson T, Zhu Y, Kirkpatrick R, Zhang GF, Johanson K, Sung CM, Liu R, Winkler J (2000) Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. J Biol Chem 275:15254-15264.

9. Karwowska-Desaulniers P, Ketko A, Kamath N, Pflum MKH (2007) Histone deacetylase 1 phosphorylation at S421 and S423 is constitutive *in vivo,* but dispensable *in vitro*. Biochem Biophys Res Commun 361:349-355.

10. Li J, Staver MJ, Curtin ML, Holms JH, Frey RR, Edalji R, Smith R, Michaelides MR, Davidsen S, Glaser KB (2004) Expression and functional characterization of recombinant human HDAC1 and HDAC3. Life Sci 74:2693-2705.

11. Rao KS, Prakash V (1993) Interaction of sodium dodecyl sulfate with multi-subunit proteins. A case study with carmin. J Biol Chem 268:14769-14775.

12. Shevchenko A, Tomas H, Havliš J, Olsen JV, Mann M (2007) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856-2860.

FIGURE LEGENDS

FIGURE 1

(A) SDS-PAGE of total proteins extracted from *E. coli* TOP10/pBAD-rHDAC1 cultures. Lane M: protein molecular mass markers (ThermoFisher Scientific). Lane NI: proteins extracted from not induced cultures. Lanes 1 and 2: proteins extracted from cultures induced with 1.3 mM arabinose for 15 h at 30 or 37 °C, respectively. The arrow indicates the band corresponding to recombinant HDAC1 (approximately 55 kDa). (B) Kinetics of growth of *E. coli* TOP10 cultures containing the pBAD empty vector (triangles) or the pBAD-rHDAC1 vector (squares) under non-inducing (empty symbols) or inducing (filled symbols) conditions, respectively. Cultures were grown in LB medium at 37 °C before induction, then the temperature was shifted to 30 °C after the addition of 1.3 mM arabinose.

FIGURE 2

(A) Purification of rHDAC1. Gel filtration chromatographic profile (Superdex 200 column) of rHDAC1 after anion exchange and affinity chromatographies (HiTrap Heparin followed by a Poly-L-lysine column). The volume of each collected fraction was 0.9 mL. (B) SDS-PAGE (10% polyacrylamide) of eluted gel filtration fractions. Lane M: molecular mass markers (116, 66, 45, 35,

25 kDa); lane I: sample of pooled fractions (input); numbered lanes: eluted fractions. (C) Stability of rHDAC1. Lane M: molecular mass markers; lanes 1-2: purified rHDAC1 incubated for 3 h at 4 and 37 °C, respectively. The arrow indicates the band corresponding to recombinant HDAC1 (55 kDa).

FIGURE 3

(A) Analysis of protein aggregates by native gel electrophoresis. Lanes 1 and 9: rHDAC1 incubated with 2% SDS; lanes 2-8 and 10-17: incubation with 500 mM KCl, 500 mM LiCl, 200 mM $MgCl_2$, 200 mM $CaCl_2$, 200 mM $SrCl_2$, 1 M urea, 10 mM β-mercaptoethanol, 1% Triton X-100, 1% Tween 20, 2% ethanol, 2% 2-propanol, 1.5% 1-butanol, 0.6% 1-pentanol, 200 mM arginine, and 200 mM betaine, respectively. (B) Screening of agents used to dissociate rHDAC1 aggregates. After incubation and filtration, samples were subjected to SDS-PAGE. Lane M: molecular mass marker; lanes 1-2: retentate and filtrate of untreated rHDAC1; lanes 3-12: retentate (odd lanes) and filtrate (even lanes) after incubation with SDS (2%, lanes 3 and 4), Triton X-100 (1%, lanes 5 and 6), Tween 20 (1%, lanes 7 and 8), urea (1 M, lanes 9 and 10), $MgCl_2$ (200 mM, lanes 11 and 12). (C) SDS-PAGE of aliquots of retentate and filtrate samples separated by Amicon Ultracel (YM100). Lane M: molecular mass markers. Lanes 1 and 4: untreated rHDAC1. Lanes 2-3: rHDAC1 samples incubated with 0.2% SDS; lanes 5-6: rHDAC1 samples incubated with 0.05% SDS.

FIGURE 4

rHDAC1 activity assay. The release of [^{3}H]acetic acid from [^{3}H]acetyl histones was assayed at 37 °C. Reaction mixtures containing 1.5 or 2.5 μg of rHDAC1 were incubated for 2 h; the reactions were then stopped, and the radioactivity associated with the release of [^{3}H]acetic was estimated. Two control reaction mixtures (containing only buffer, and devoid of enzyme), were also assayed. One of these control reaction mixtures was also supplemented with 0.2% SDS (filled triangle).

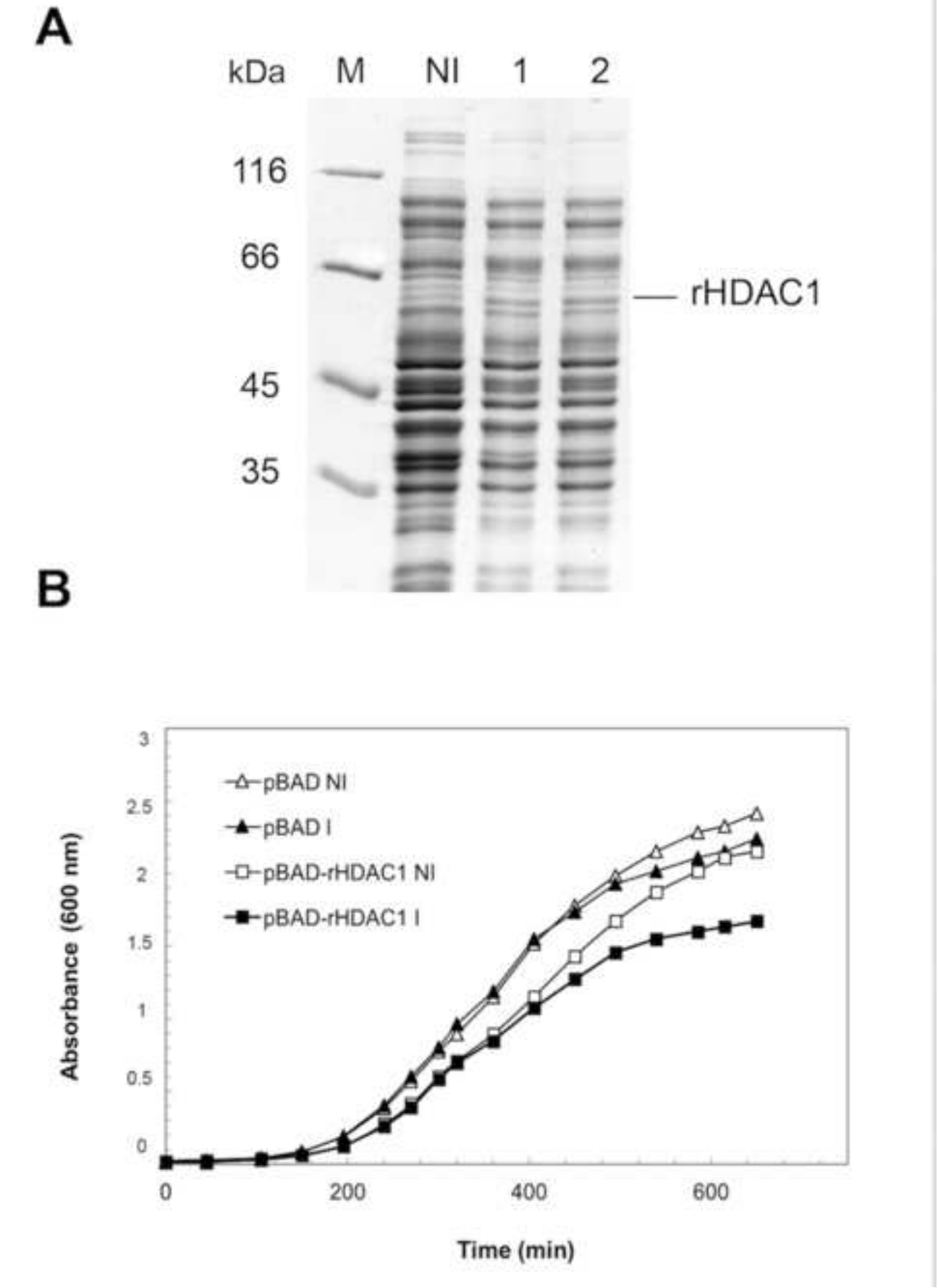
1

2

3

4

Figure 1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rHDAC1
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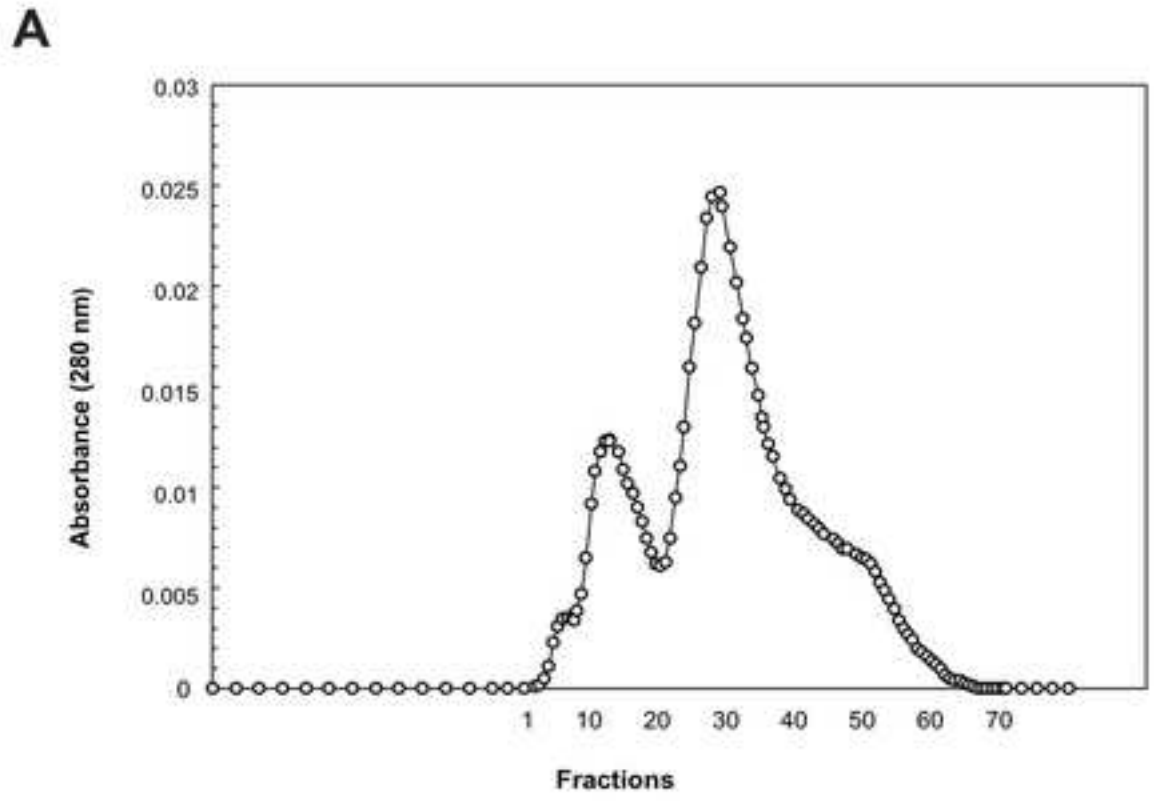
Figure 2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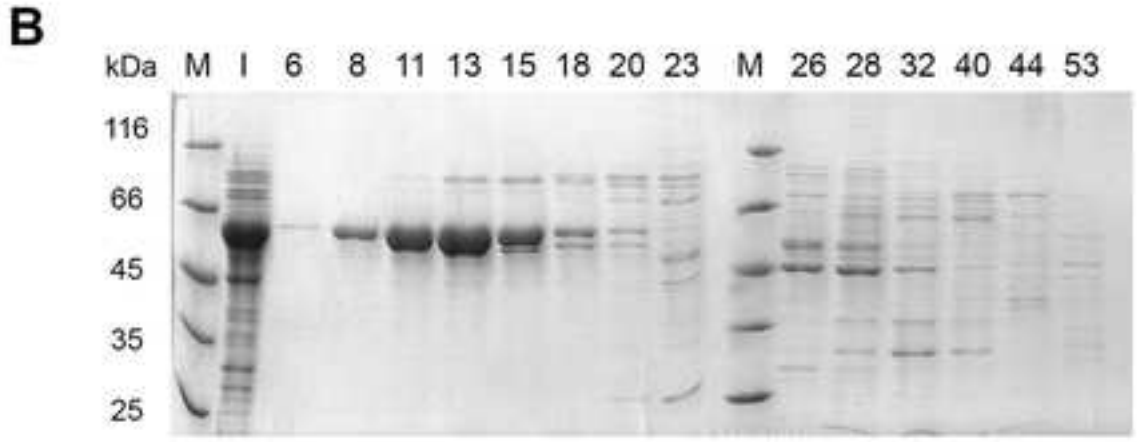
Fractions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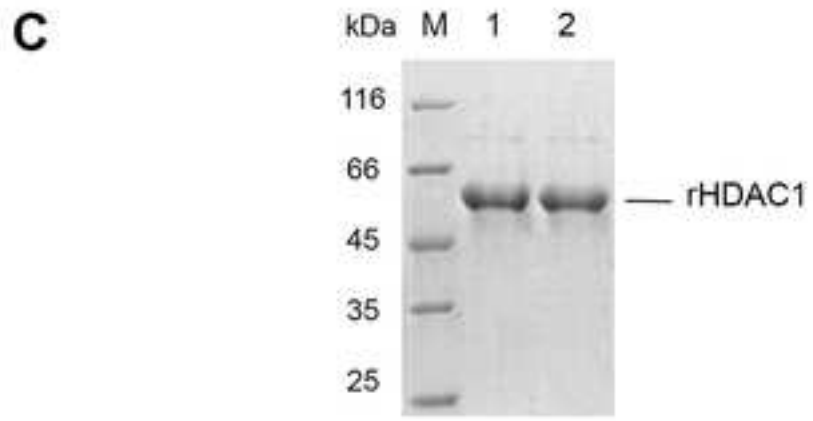
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Figure 3

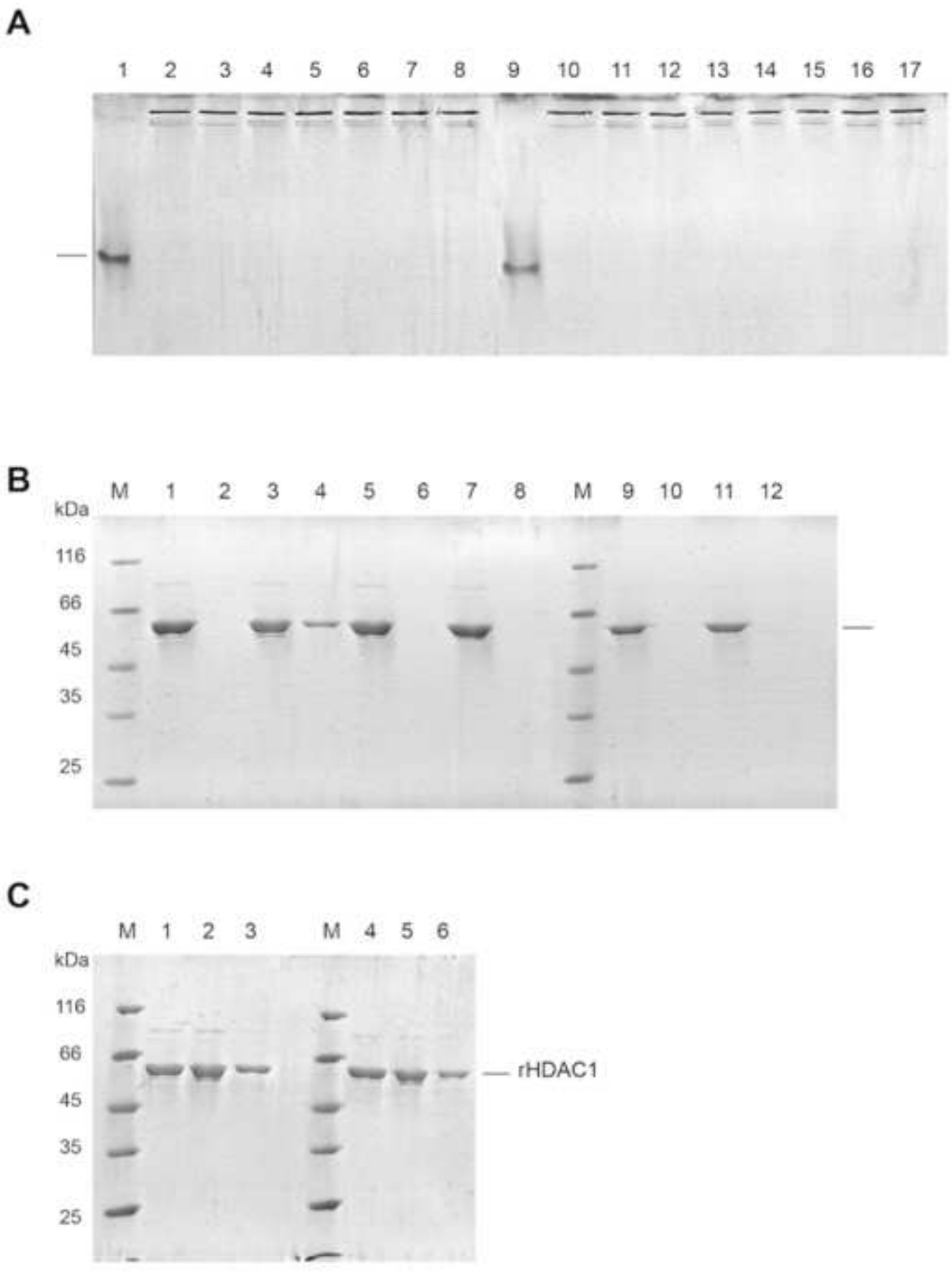
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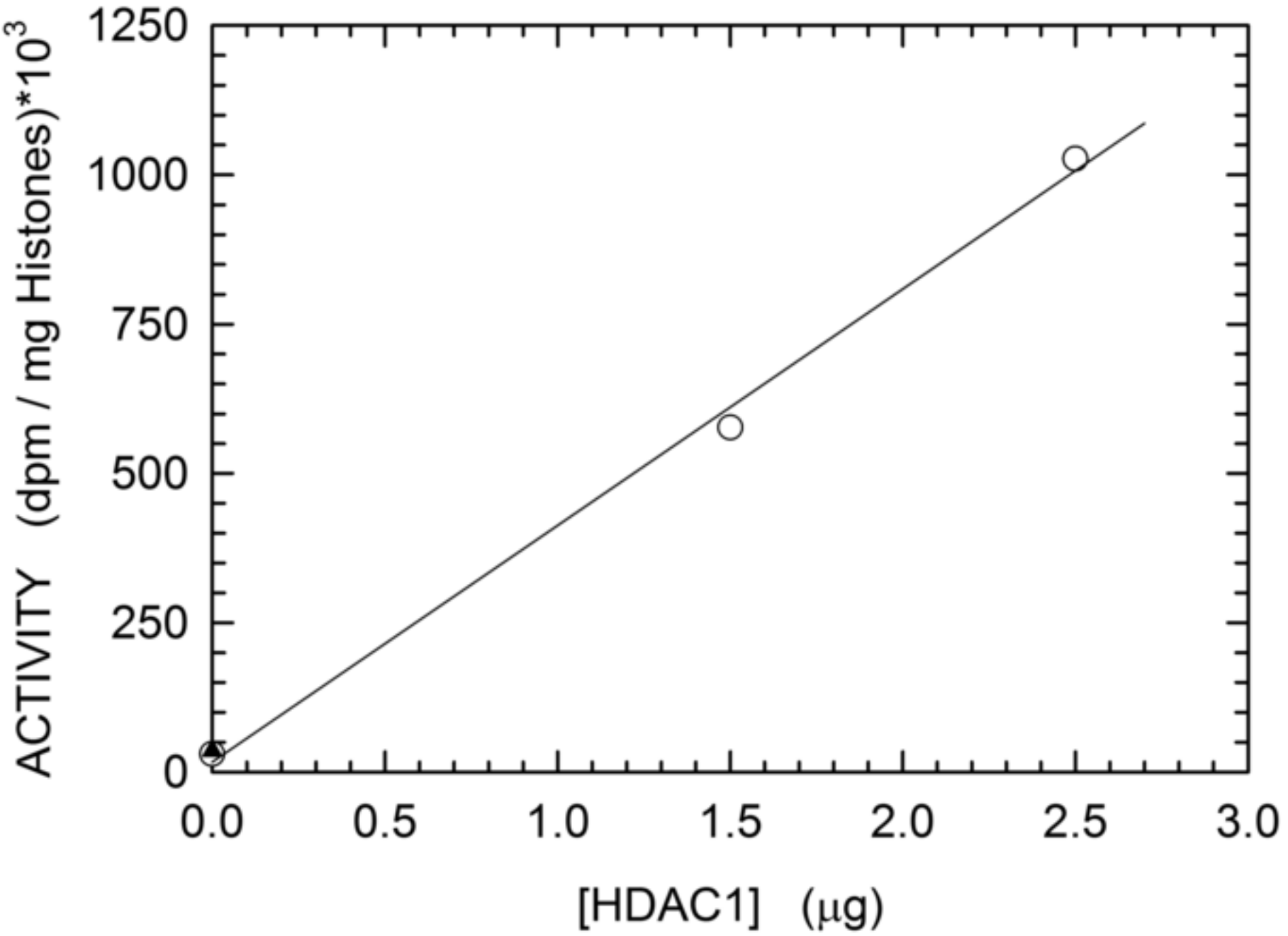
Figure 4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750
500
250
0
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0.5
1.0
1.5
2.0
2.5
3.0
[HDAC1] (µg)

TABLE 1. Mass spectrometry data leading to the identification of HDAC1 peptides following in gel digestion of the purified protein. The mass/charge (m/z) ratio of the detected ions is indicated, along with the corresponding identified fragments (peptide) of HDAC1, whose detected (observed) and expected (theoretical) masses are reported in Dalton.

Observed ion (*m/z*)	Molecular mass (observed)	Peptide	Molecular mass (theoretical)	Difference (ppm)
588.6564	2938.2556	11–34	2938.2592	-1.23
626.3444	1876.0114	35–49	1876.0141	-1.46
804.4219	1606.8292	37–49	1606.8289	0.19
579.3143	1734.9211	37–50	1734.9239	-1.63
537.2816	1072.5486	51–58	1072.5488	-0.097
638.9561	1913.8465	59–74	1913.8465	-0.013
520.7377	1039.4608	67–74	1039.4611	-0.2
476.2258	1425.6556	78–89	1425.6558	-0.15
1079.8342	3236.4808	94–123	3236.4795	0.39
615.9831	1844.9275	127–143	1844.9282	-0.38
828.113	2481.3172	144–165	2481.3189	-0.68
1177.6198	2353.225	145–165	2353.2239	0.48
735.3841	2937.5073	145–169	2937.5058	0.5
893.4291	2677.2655	170–192	2677.266	-0.21
482.7491	963.4836	193–200	963.4848	-1.2
687.8198	1373.625	201–212	1373.6252	-0.098
579.7999	1157.5852	221–229	1157.5869	-1.44
800.637	3198.5189	221–247	3198.5219	-0.95
1022.4819	2042.9492	230–247	2042.9507	-0.69
1257.0758	2512.137	248–270	2512.1396	-1.03
533.2903	1064.566	271–279	1064.5688	-2.63
913.967	1825.9194	290–306	1825.9219	-1.34
1117.0306	2232.0466	343–361	2232.048	-0.63
1211.2098	3630.6076	372–404	3630.609	-0.39
455.2207	908.4268	405–412	908.4273	-0.52
579.2526	2312.9813	413–432	2312.9815	-0.084
719.9678	2156.8816	414–432	2156.8804	0.55
492.8951	1475.6635	445–456	1475.6627	0.52

TABLE 2. Additives used to promote protein dissociation. The concentrations indicated are expressed as: % (v/v) for detergents and alcohols, mM for salts, urea, and β-mercaptoethanol, and μM for fatty acids.

Reagent	Concentration
Sodium dodecyl sulfate	2
Triton X-100	1
Tween 20	1
KCl	500
LiCl	500
$MgCl_2$	200
$CaCl_2$	200
$StCl_2$	200
Urea	1000
Arginine	200
Betaine	200
Glycine	200
Ethanol	2
2-propanol	2
1-butanol	1.5
1-pentanol	0.6
β-mercaptoethanol	10
Lauric acid	200
Myristic acid	200
Stearic acid	200