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Supplementary Information for

Unique subsite specificity and potential natural function of a chitosan deacetylase from the human pathogen Cryptococcus neoformans

Lea Hembach [a], Martin Bonin [a], Christian Gorzelanny [b], Bruno M. Moerschbacher [a,1]

Bruno M. Moerschbacher
Email: moersch@uni-muenster.de

This PDF file includes:

Figures S1 to S6
Table S1

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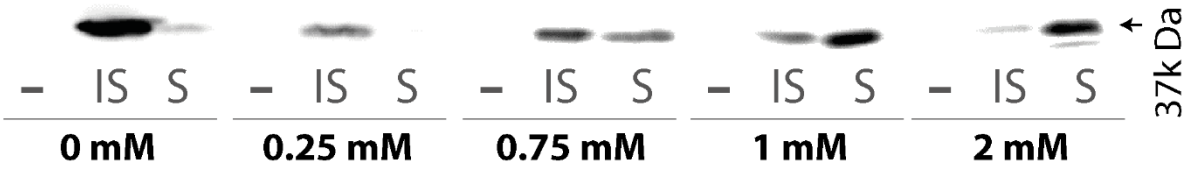

Fig. S1. Comparison of separated cell fractions of CnCda4 expressed in E. coli Lemo21 cells in the presence of α-L-rhamnose (0–2 mM) by immunoblotting using an HRP coupled Strep-tag II affinity protein. (–) Before induction of expression with 400 µM IPTG, (IS) insoluble fraction, (S) soluble fraction.

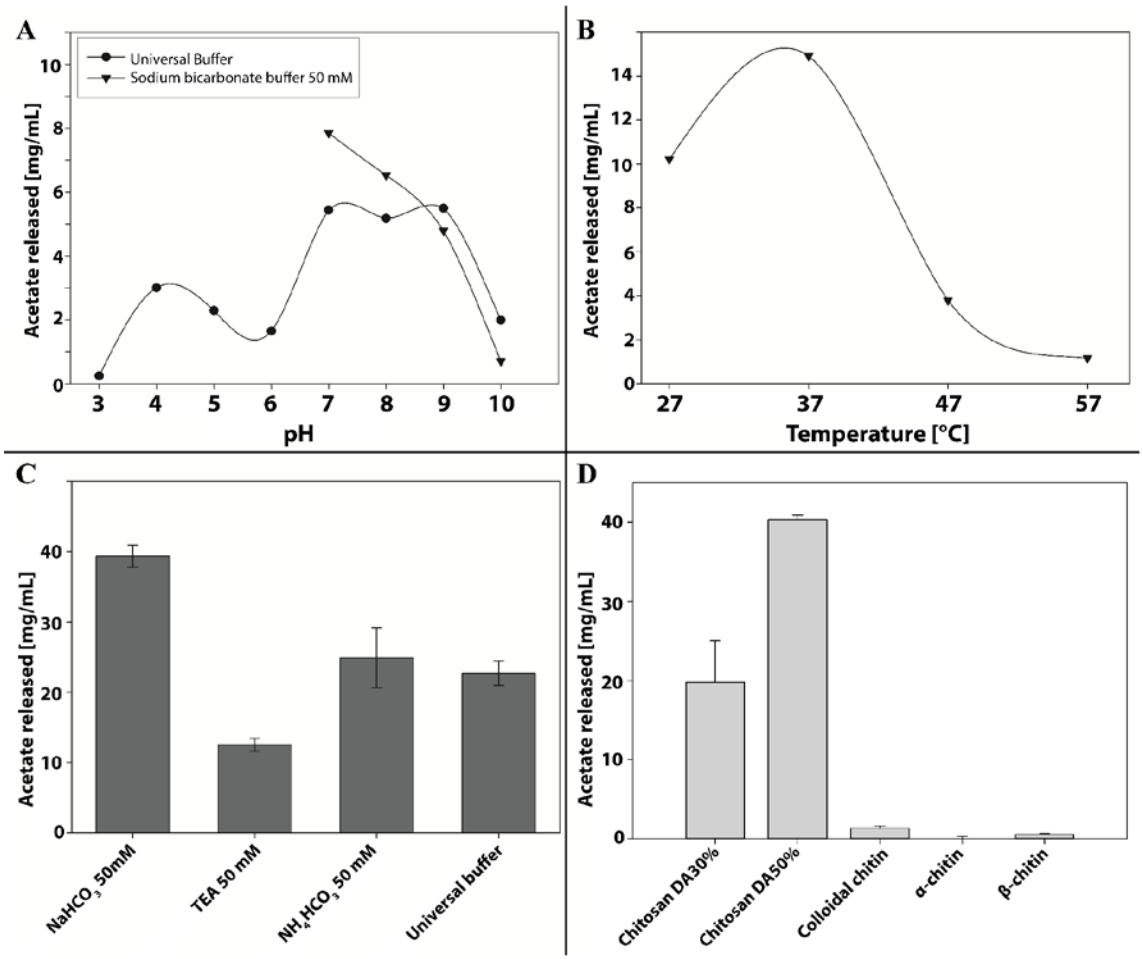

Fig. S2. CnCda4 in terms of pH, temperature, reaction buffer, and substrate. Enzyme activity was calculated from the quantity of acetate released during the reaction and each experiment was carried out at least in duplicate. (*A*) The pH and (*B*) temperature optima were determined by incubating 0.5 µg CnCda4 for (*A*) 20 h or (*B*) 22 h at 37 °C with chitosan DA50%. (*A*) Sodium bicarbonate buffer (50 mM) ranging from pH 7 to 10 and a universal buffer consisting of 20 mM CH_5NO_2, 10 mM TEA, 10 mM KH_2PO_4, and 10 mM Na_2HPO_4, (pH 3–10). (*B*) 50 mM $NaHCO_3$, pH 7. (*C*) We incubated 1 µg CnCda4 for 24 h with chitosan DA50% at 37 °C in 50 mM $NaHCO_3$, 50 mM pH7, TEA (50 mM), NH_4HCO_3 (50 mM), and universal buffer. (*D*) We incubated 20 µL of CnCda4 with chitosan DA50%, colloidal chitin, *α*-chitin and *β*-chitin for 24 h at 37 °C in 50 mM $NaHCO_3$ (pH7). For *C* and *D*, n = 3, mean ± SD.

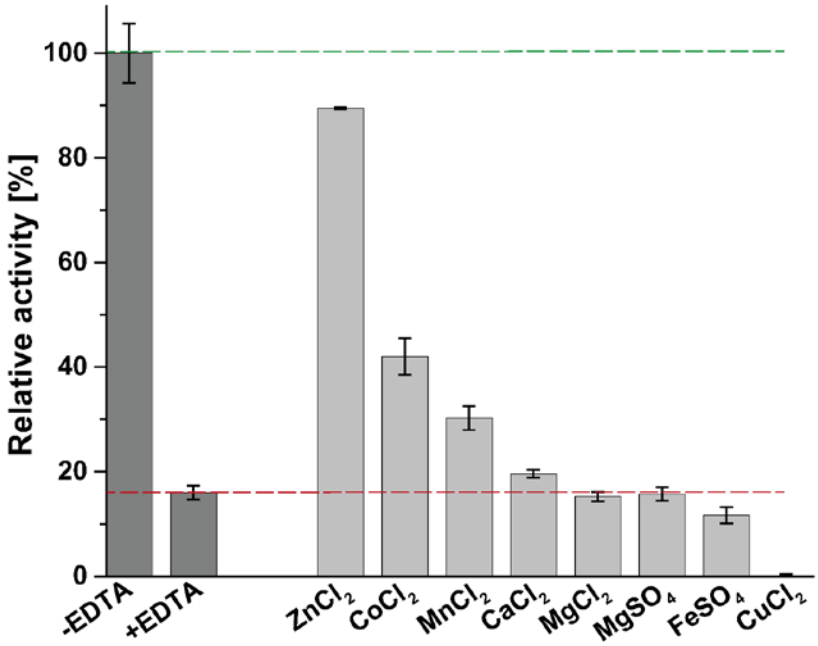

Fig. S3. Relative CnCda4 activity incubated without (–) EDTA and with (+) EDTA, before dialysis against $NaHCO_3$ to remove all EDTA and subsequent incubation with 1 mM metal salts for 2 h prior to activity tests on A4. The activity was measured by integrating LC-MS peak areas (n = 3, mean ± SD).

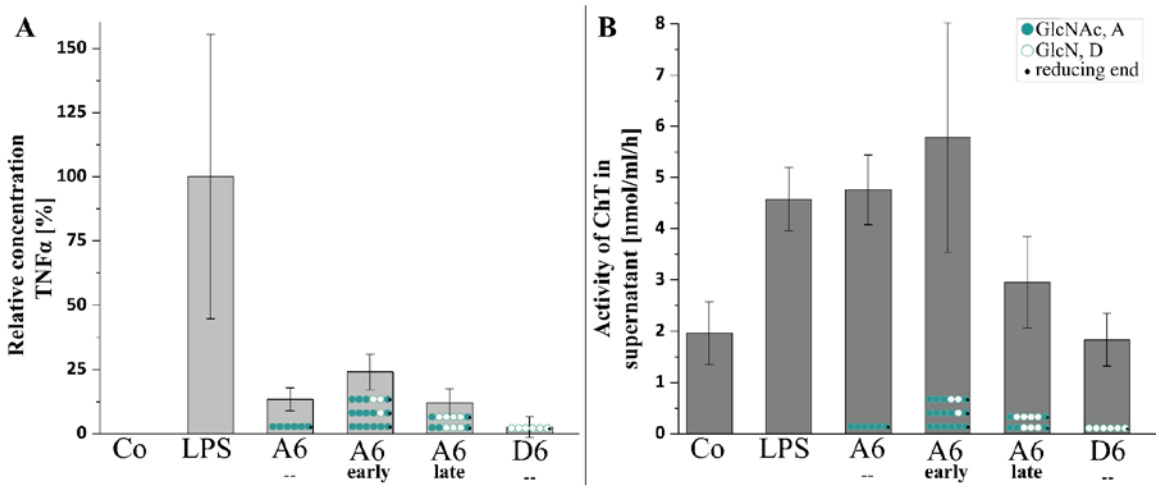

Fig. S4. The effect of early and late CnCda4 products, A6/D6 controls, culture medium as negative control, and LPS as positive control on the secretion of TNFα and the activity of the human chitotriosidase (ChT) in the cell supernatant of human peripheral blood-derived macrophages (HPBMs). (*A*) Activation of HPBMs by different chitin and chitosan hexamers (1 µg/mL). Secretion of TNFα into the supernatant was measured 6 h after stimulation. Data from five independent experiments with different batches of macrophages were normalized to the negative control (starvation medium, 0%) and to the positive control (10 ng/mL LPS, 100%). Data are presented as means ± SD. (*B*) Activity of the human ChT measured in the supernatant after stimulation with the different chitin and chitosan hexamers (1 µg/mL). The concentration of ChT in 5 µL supernatant was determined by adding 20 mM of the ChT substrate 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside in 100 mL McIlvain buffer (100 mM citric acid, 200 mM sodium phosphate, pH 5.2). The enzyme reaction was stopped after 20 min at 37 °C by adding 200 mL 300 mM glycine-NaOH buffer, pH 10.5. Converted substrate was measured by fluorimetry at 450 nm and related to a standard of recombinant ChT. Values are means of three independent experiments ± SD.

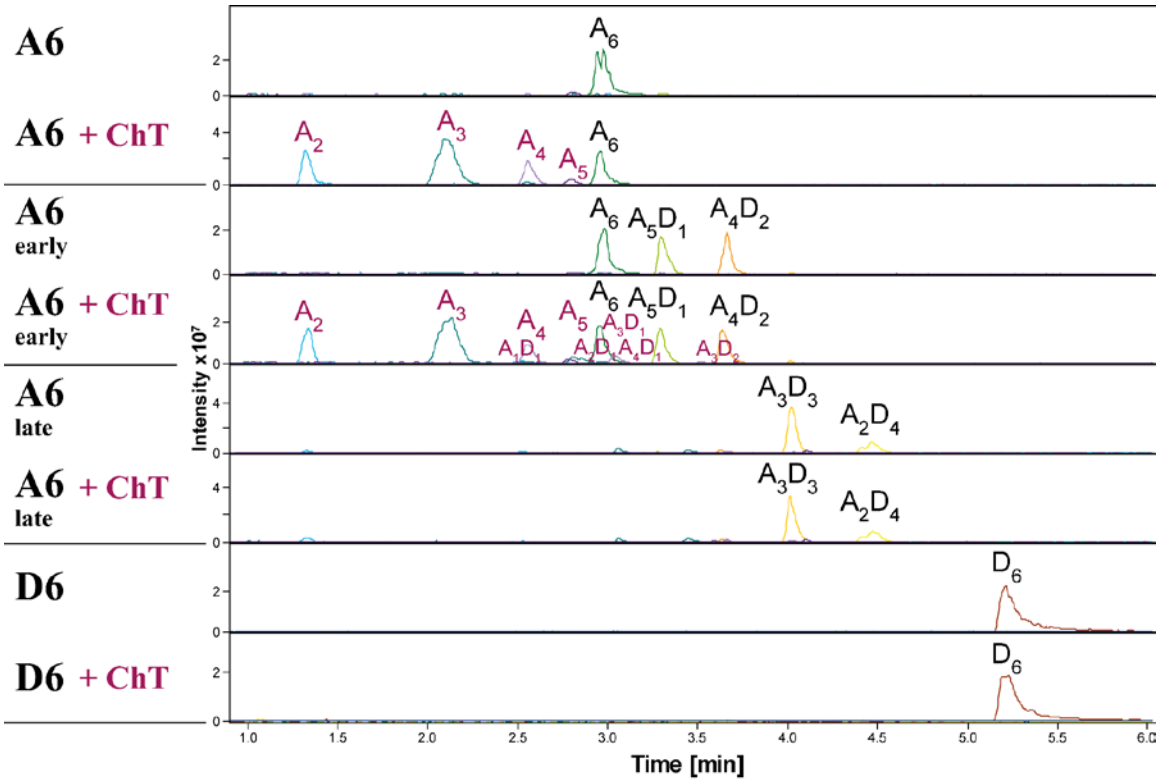

Fig. S5. Product profile following the digestion of hexameric CnCda4 products (used for stimulation of human macrophages) with human chitotriosidase (ChT). We incubated 5 µg of the hexameric samples with 50 ng of purified ChT for 24 h at 37 °C in 50 mM ammonium acetate buffer pH (4.5). The products were quantified by HPLC-MS analysis.

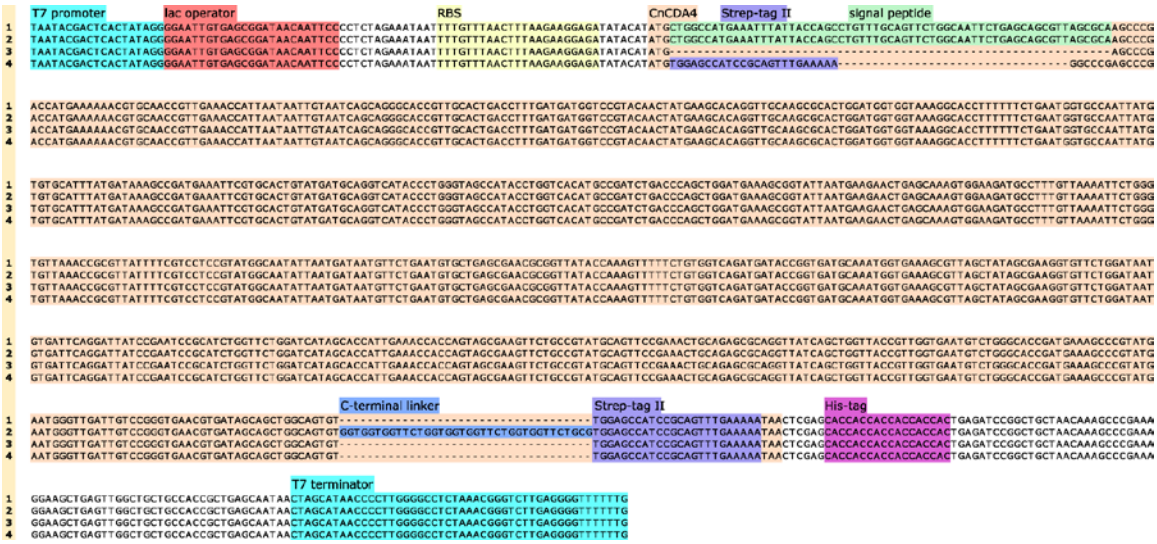

Fig. S6. Sequence alignment of the different CnCda4 constructs used during this work. (1) pET22b(+)::SP-*CDA4*-StrepII, (2) pET22b(+)::SP-*CDA4*-linker-StrepII, (3) pET22b(+)::*CDA4*-strepII, and (4) pET22b(+)::StrepII-*CDA4*-StrepII. All constructs have a pET22b(+) backbone differing only in their sequences shown in this alignment, which contains the following elements: T7 promoter, *lac* operator, ribosome binding site (RBS), CnCda4 sequences including either an N-terminal Strep-tag II or the original signal peptide (SP), a C-terminal linker and a C-terminal Strep-tag II, an unused His_6 tag originating from the pET22b(+) plasmid and the T7 terminator.

Table S1. Mono-isotopic *m/z* values of chitin and chitosan oligomers used for analysis, isolation and fragmentation (MS/MS) with A representing an *N*-acetyl-D-glucosamine unit, D a D-glucosamine unit, R a D-glucosamine unit chemically acetylated using [$^{2}H_{6}$]acetic anhydride (Sigma-Aldrich) and ^{18}O representing the reducing end labeled with $H_2{}^{18}O$.

AD	H+ Charge1	H+ Charge2	AD ^{18}O	H+ Charge1	H+ Charge2	AR	H+ Charge1	H+ Charge2	AR ^{18}O	H+ Charge1	H+ Charge2
A1	222.097		**A1**	224.097		**A1**	222.097		**A1**	224.101	
D1	180.087		**R1**	182.087		**R1**	225.116		**R1**	227.120	
A2	425.177	213.092	**A2**	427.177	214.092	**A2**	425.177	213.092	**A2**	427.181	214.094
A1D1	383.166	192.087	**A1R1**	385.166	193.087	**A1R1**	428.195	214.601	**A1R1**	430.200	215.603
D2	341.155	171.081	**R2**	343.155	172.081	**R2**	431.214	216.111	**R2**	433.218	217.113
A3	628.256	314.632	**A3**	630.256	315.632	**A3**	628.256	314.632	**A3**	630.260	315.634
A2D1	586.245	293.626	**A2R1**	588.245	294.626	**A2R1**	631.275	316.141	**A2R1**	633.279	317.143
A1D2	544.235	272.621	**A1R2**	546.235	273.621	**A1R2**	634.294	317.650	**A1R2**	636.298	318.653
D3	502.224	251.616	**R3**	504.224	252.616	**R3**	637.312	319.160	**R3**	639.317	320.162
A4	831.335	416.171	**A4**	833.335	417.171	**A4**	831.335	416.171	**A4**	833.340	417.173
A3D1	789.325	395.166	**A3R1**	791.325	396.166	**A3R1**	834.354	417.681	**A3R1**	836.358	418.683
A2D2	747.314	374.161	**A2R2**	749.314	375.161	**A2R2**	837.373	419.190	**A2R2**	839.377	420.192
A1D3	705.304	353.155	**A1R3**	707.304	354.155	**A1R3**	840.392	420.700	**A1R3**	842.396	421.702
D4	663.293	332.150	**R4**	665.293	333.150	**R4**	843.411	422.209	**R4**	845.415	423.211
A5	1034.415	517.711	**A5**	1036.415	518.711	**A5**	1034.415	517.711	**A5**	1036.419	518.713
A4D1	992.404	496.706	**A4R1**	994.404	497.706	**A4R1**	1037.434	519.220	**A4R1**	1039.438	520.223
A3D2	950.394	475.700	**A3R2**	952.394	476.700	**A3R2**	1040.452	520.730	**A3R2**	1042.457	521.732
A2D3	908.383	454.695	**A2R3**	910.383	455.695	**A2R3**	1043.471	522.239	**A2R3**	1045.475	523.241
A1D4	866.372	433.690	**A1R4**	868.372	434.690	**A1R4**	1046.490	523.749	**A1R4**	1048.494	524.751
D5	824.362	412.685	**R5**	826.362	413.685	**R5**	1049.509	525.258	**R5**	1051.513	526.260
A6	1237.494	619.251	**A6**	1239.494	620.251	**A6**	1237.494	619.251	**A6**	1239.498	620.253
A5D1	1195.484	598.245	**A5R1**	1197.484	599.245	**A5R1**	1240.513	620.760	**A5R1**	1242.517	621.762
A4D2	1153.473	577.240	**A4R2**	1155.473	578.240	**A4R2**	1243.532	622.270	**A4R2**	1245.536	623.272
A3D3	1111.462	556.235	**A3R3**	1113.462	557.235	**A3R3**	1246.551	623.779	**A3R3**	1248.555	624.781
A2D4	1069.452	535.230	**A2R4**	1071.452	536.230	**A2R4**	1249.569	625.288	**A2R4**	1251.574	626.290
A1D5	1027.441	514.224	**A1R5**	1029.441	515.224	**A1R5**	1252.588	626.798	**A1R5**	1254.592	627.800
D6	985.431	493.219	**R6**	987.431	494.219	**R6**	1255.607	628.307	**R6**	1257.611	629.309