Supplementary Information

Computational thermostability engineering of a nitrile hydratase using Synergetic Energy and Correlated-configuration for Redesigning Enzyme (SECURE) Strategy

Jinling Xu[1], Haisheng Zhou[2], Jiaqi Xu[2]*, Ziyuan Wang[1], Zhonglang Yu[1], Zhe Wang[1], Hongyu Zhang[1,2], Haoran Yu[1,2], Jianping Wu[1,2], Lirong Yang[1,2]*

[1] *Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou, 310027, China.*

[2] *ZJU-Hangzhou Global Scientific and Technological Innovation Centre, NO.733 Jianshe 3rd Road, Xiaoshan District, Hangzhou, Zhejiang, 311200, China.*

* Corresponding author: E-mail of Jiaqi Xu: biojqxu@zju.edu.cn, E-mail of Lirong Yang: lryang@zju.edu.cn

Additional tables

Table S1. NHase library.

Entry	NHase	Organism	Accession ID
1	NHAB	*Bordetella petrii* strain DSM12804	AM902716.1
2	NHAK	*Klebsiella oxytoca* KCTC1686	AEX05823.1
3	NHAP	*Pseudomonas putida* P38K	U89363.2
4	NHAC	*Comamonas testosteroni* strain 5-MGAM-4D	AY743666.1
5	NHPT	*Pseudonocardia thermophila* JCM3095	1IRE
6	NHCTA	*Caldakalibacillus thermarum*	WP_007502644
7	NHAM	*Aurantimonas* manganoxydans *strain* S185-9A1	KP236110.1
8	NHNO	*Nocardia* sp.	AY168347.1
9	NHJL	*Rhodococcus rhodochrous*	X64360.1
10	NHJH	*Rhodococcus rhodochrous*	D67027.1
11	NHTH	*Rhodococcus ruber* TH3	RQM32616.1
12	M33	*Rhodococcus rhodochrous* M33	DI030052.1
13	NHRP	*Ruegeria pomeroyi* DSS-3	CP000031.2
14	NHBO	*Bradyrhizobium oligotrophicum* S58	AP012603.1
15	NHPAT	*Parageobacillus thermoglucosidasius*	WP_125010986.1
16	NHPAA	*Paenibacillus abyssi* strain CGMCC 1.12987	WP_188529557.1
17	NHTM	*Thermoactinomyces* sp. CICC 10522	WP_198064934.1
18	NHAD	*Aneurinibacillus danicus* strain NBRC 102444	WP_146809496.1
19	NHGT	*Geobacillus thermoleovorans*	WP_081130963.1

Table S2. Unreasonable destabilizing mutations with presumed reason for elimination.

Mutations	Presumed reason for elimination
• α-subunit: M43L; V92R; • β-subunit: A10Q, G27A, P38L, I61A, T67S, I76L, S119P, K135R;	Mutations with unfavorable mutation energy: $\Delta\Delta G > 0$
• α-subunit: F110V, N157W, Y184W; • β-subunit: G94R, K99L, G112W, K123P, D163V;	Mutations would destruct the secondary structure or break the molecular interactions
• α-subunit: Y14H, L23A, K53E, V54W, L100V, T201V; • β-subunit: V33A, T102K, T102P, H180N, I206V, V208I, D212E	Mutations located in most rigid region: the lowest Ri
• α-subunit: A45L/R, V46L/I, V47I, Q48E, N79D, G83A, A105D, T156D, K158V; • β-subunit:, G66S/A, T67P, L86I, T93Q, A96Q/G, L113F, S115A, A120C, L139R, V144A, T159V, V160I, V184L, S191A, Q197E, S200A/G, S201F;	Mutations had poor correlation with active center: -0.1< cij<0.1
• α-subunit: A179P; • β-subunit: S75G;	Similar mutations were chosen for experimental verification.

Table S3. Specific activity of pure enzyme.

Mutants	Specific activity (U/mg protein)
Wild type	1510±72
A6M	1753±46
B4M	1773±182
A6M/B4M	1697±92

Table S4. Product tolerance of cells.

Mutants	Residual activity (%)
Wild type	0
A6M/B4M	38.4

Table S5. The parameters set using FIREPROT.

Parameter setting	Input
Source	Construct new alignment
Blast E-value	$1*10^{-10}$
Minimal identity (%)	30
Max. number of sequences	200
Maximal identity (%)	90

Table S6. The parameters set using PROSS.

Parameter setting	Input
PDB chain	A/B
Small-molecule ligands	No
Interacting chains	B/A
Minimal sequence identity	35
Coverage	75
Maximum targets	3000
E-value	0.0001

Table S7. Sequences of Primers.

Primers	Sequence (5' to 3')
A-S30T-F	TTGGAGaccCTGCTCGTCGAGAAAGGTTTGGT
A-S30T-R	ACGAGCAGggtCTCCAAGGCCTTCACCCGCAG
A-A71D-F	TGGACCCGgacTACAAGGCGCGCTTGCTGGCG
A-A71D-R	CTTGTAgtcCGGGTCCACCCAGGCCTTGGCAA
A-A74D-F	ATACAAGgacCGCTTGCTGGCGAATGGCAGCG
A-A74D-R	GCAAGCGgtcCTTGTATGCCGGGTCCACCCAG
A-A78R-F	CTTGCTGcgcAATGGCAGCGCTGGCATTGCCG
A-A78R-R	TGCCATTgcgCAGCAAGCGCGCCTTGTATGCC
A-S81T-F	AATGGCaccGCTGGCATTGCCGAACTGGGCTT
A-S81T-R	ATGCCAGCggtGCCATTCGCCAGCAAGCGCGC

A-D96H-F	GGGAGAAcacACAGTCATTCTGGAAAACACCCC
A-D96H-R	TGACTGTgtgTTCTCCCTGCACTCCAGAGAAG
A-T97M-F	GGAGAAGACatgGTCATTCTGGAAAACACCCCC
A-T97M-R	ATGACcatGTCTTCTCCCTGCACTCCAGAGAA
A-S122V-F	TACCCATGGCCGgtgCTGGGCTTGCCGCCGGCC
A-S122V-R	AGcacCGGCCATGGGTAGCAAGAGCACAGGGT
A-A132S-F	TACAAGtcgGCACCCTACCGGTCGCGCATGGT
A-A132S-R	TAGGGTGCcgaCTTGTACCAGGCCGGCGGCAA
A-A133P-F	TACAAGGCCccgCCCTACCGGTCGCGCATGGT
A-A133P-R	TAGGGcggGGCCTTGTACCAGGCCGGCGGCAA
A-T165S-F	GACtcgACAGCCGAATTGCGCTACATGGTGCT
A-T165S-R	AATTCGGCTGTcgaGTCCCAGACGCGGATTTCC
A-T166S-F	GACACCtcgGCCGAATTGCGCTACATGGTGCT
A-T166S-R	AATTCGGCcgaGGTGTCCCAGACGCGGATTTC
A-A167S-F	CACCACAtcgGAATTGCGCTACATGGTGCTGC
A-A167S-R	GCAATTCcgaTGTGGTGTCCCAGACGCGGATT
A-A179E-F	gaaGGAACCGAAGGCTACAGCGAAGAACAACT
A-A179E-R	TAGCCTTCGGTTCCttcGGGCCTTTCCGGCAGCAC
A-Q188E-F	GAAGAAgaaCTGGCCGAACTCGTCACCCGCGA
A-Q188E-R	TCGGCCAGttcTTCTTCGCTGTAGCCTTCGGT
B-T7L-F	ACGGCATTCACGACctgGGCGGAGCACATGGTTATGG
B-T7L-R	cagGTCGTGAATGCCGTTCATGATGATCTCCT
B-Y13F-F	ATGGTtttGGCCCGGTTTACAGGGAGCCGAAT
B-Y13F-R	AACCGGGCCaaaACCATGTGCTCCGCCAGTGT
B-V16I-F	CGGAGCACATGGTTATGGCCCGATCTACAGGGAGC
B-V16I-R	GGCTCATTCGGCTCCCTGTAGATCGGGCCATAACC
B-N21D-F	GGCCCGGTTTACAGGGAGCCGGACGAGCCCATCCT
B-N21D-R	CGCCATGAAGGATGGGCTCGTCCGGCTCCCTGTAA
B-L25F-F	AATGAGCCCATCTTCCATGGCGAGTGGGAGGGTCG
B-L25F-R	CAGGACCCGACCCTCCCACTCGCCATGGAAGATGG
B-G27Y-F	CCTTCATtacGAGTGGGAGGGTCGGGTCCTGG
B-G27Y-R	CCCACTCgtaATGAAGGATGGGCTCATTCGGC
B-G54A-F	ACATCGATGAGTTTCGACACGCAATCGAGCGCATG
B-G54A-R	ATGGGGTTCATGCGCTCGATTGCGTGTCGAAACTC
B-I55R-F	TTTCGACACGGCcgaGAGCGCATGAACCCCATCG
B-I55R-R	TCtcgGCCGTGTCGAAACTCATCGATGTTGAA
B-N59P-F	ATGccgCCCATCGACTACCTGAAGGGAACCTA
B-N59P-R	TAGTCGATGGGcggCATGCGCTCGATGCCGTG
B-S75A-F	GGATCCATgcaATCGAAACCTTGCTGGTCGAA

B-S75A-R	TTCGATtgcATGGATCCAGTGTTCGTAGTAGGTT
B-G112A-F	GACgcaCTGCTCAGTAACGGAGCTTCTGCCGC
B-G112A-R	TTACTGAGCAGtgcGTCCACCATGACCGGCGT
B-Q127P-F	AAGGAGGGGGTGccgGCGCGGTTCGCTGTGGGC
B-Q127P-R	GCcggCACCCCCTCCTTGCGGGCGGCAGAAGC
B-A128P-F	TGCAGccgCGGTTCGCTGTGGGCGACAAGGTT
B-A128P-R	AGCGAACCGcggCTGCACCCCCTCCTTGCGGG
B-T169F-F	TGGTGTGTTCGTGttcCCGGACACCGCGGCACAC
B-T169F-R	GgaaCACGAACACACCATGGTCGATGACCACT
B-T172Y-F	tacGCGGCACACGGAAAGGGCGAGCACCCCCA
B-T172Y-R	TTTCCGTGTGCCGCgtaGTCCGGCGTCACGAACACA
B-A173N-F	ACCaacGCACACGGAAAGGGCGAGCACCCCCA
B-A173N-R	TTTCCGTGTGCgttGGTGTCCGGCGTCACGAA

Additional figures
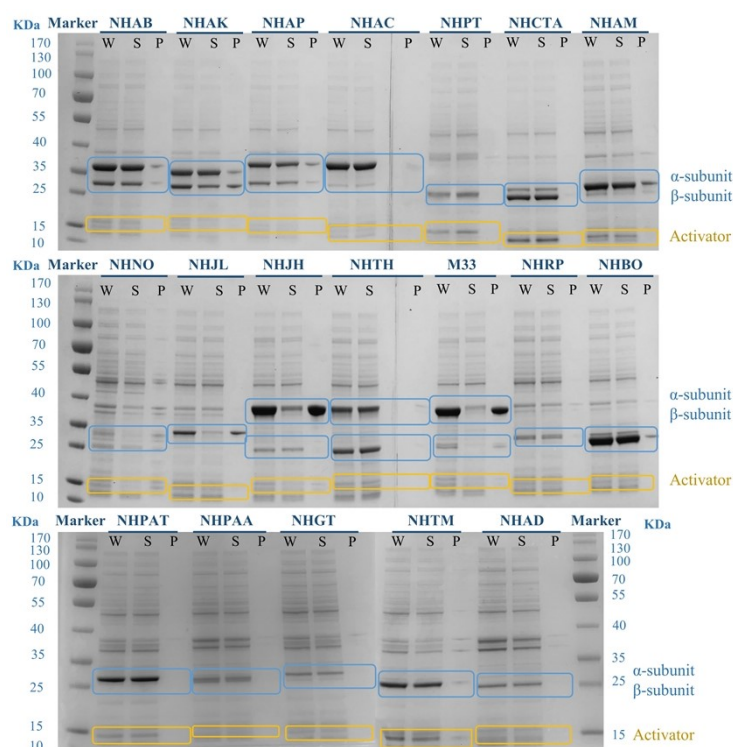
Figure S1. SDS-PAGE analysis of the protein expression of the NHases from library. W: whole cell; S: supernate; P: precipitate. The blue box represented the α and β subunits and the yellow box represents the activator. Some of the α and β subunits of NHase were overlapped on the SDS-PAGE due to the similar protein size.
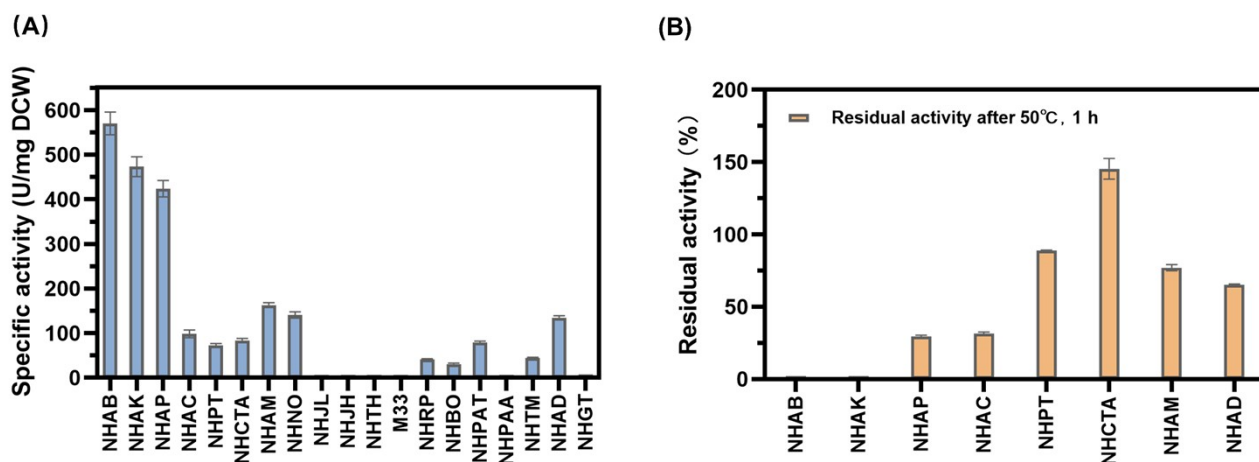
Figure S2. Enzyme activity detection of the NHase library toward acrylnitrile. (A) Initial crude enzyme activity of NHase with acrylonitrile as substrate. **(B)** Thermostability of NHase with relative high catalytic activity in the library. The residual activity was characterized after 1 h heat treatment at 50°C.
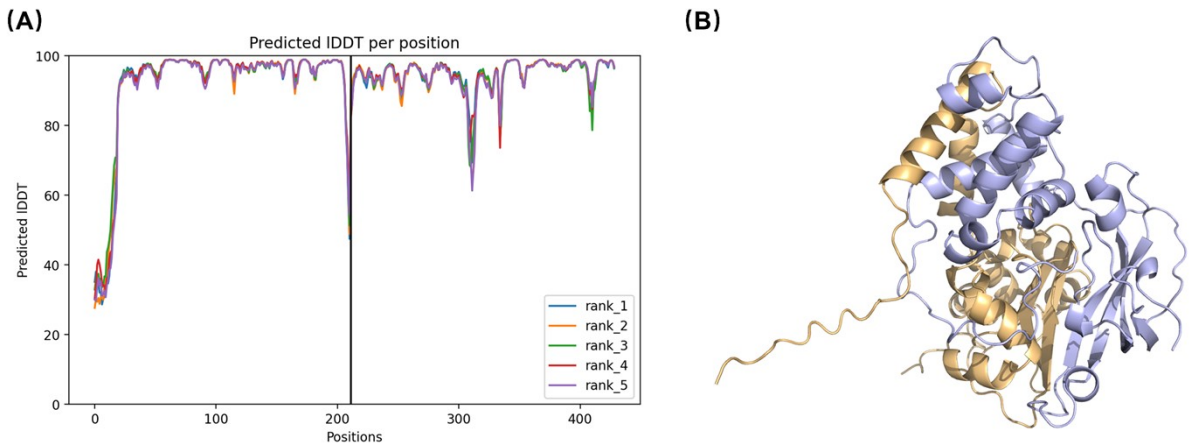
Figure S3. (A) The score result of modeling by AlphaFold2. **(B)** Protein structure with high reliability of NHAB for modification. α-subunit was painted in yellow while β-subunit was in lightblue.

(A)

| chain	position	ref	alt	conserved	correlated	foldx	rosetta
A	45	A	L	3	2.06	-1.2	-2.11
A	54	V	W	5	0.41	-2.07	-5.27
A	122	S	T	5	1.55	-1.65	-2.78
A	157	N	W	3	1.59	-1.32	-2.33
A	179	A	P	2	1.17	-1.08	-5.69
B	27	G	Y	4	-0.15	-1.53	-5.61
B	54	G	A	5	-0.17	-1.5	-4.22
B	67	T	P	5	-0.08	-1.72	-2.89
B	75	S	A	5	-0.09	-1.2	-2.5
B	93	T	Q	3	0.16	-1.17	-2.46
B	112	G	W	2	0.09	-1.44	-2.03
B	113	L	F	4	-0.45	-1.14	-2.74
B	127	Q	P	4	-0.35	-1.05	-5.85
B	172	T	Y	5	-0.25	-2.15	-2.77
B	201	S	F	4	0.11	-2.23	-4.96

(B)

| chain	position	ref	alt	majority	ratio	foldx
A	43	M	L	✓	✓	0.04
A	46	V	L	✓	✓	-0.79
A	47	V	I	✓	✗	-0.62
A	48	Q	E	✗	✓	-0.11
A	79	N	D	✓	✓	-1.87
A	92	V	R	✓	✓	0.46
A	97	T	M	✓	✓	-2.34
A	122	S	V	✓	✓	-4.11
A	132	A	S	✓	✗	-0.49
A	165	T	S	✓	✓	-0.32
A	184	Y	W	✗	✓	-0.59
B	7	T	L	✓	✓	-1.74
B	10	A	Q	✗	✓	0.14
B	13	Y	F	✓	✓	-0.53
B	25	L	F	✓	✓	-0.73
B	27	G	A	✓	✓	0.22
B	38	P	L	✓	✓	0.28
B	54	G	A	✓	✓	-1.5
B	59	N	P	✗	✓	-0.37
B	61	I	A	✓	✓	0.3
B	67	T	S	✓	✗	0.36
B	75	S	A	✓	✓	-1.2
B	76	I	L	✓	✓	0.19
B	102	T	K	✗	✓	-0.58
B	115	S	A	✓	✓	-0.28
B	119	S	P	✓	✗	0.07
B	123	K	P	✗	✓	-0.61
B	127	Q	P	✗	✓	-1.05
B	135	K	R	✓	✓	0.11
B	160	V	I	✓	✗	-0.4
B	163	D	V	✓	✗	-1.12
B	169	T	F	✓	✓	-0.36
B	173	A	N	✓	✗	-0.04
B	180	H	N	✗	✓	-0.5
B	191	S	A	✗	✓	-1.06
B	197	Q	E	✗	✓	-0.01
B	200	S	A	✓	✓	-0.3
B	208	V	I	✓	✗	-0.45

Figure S4. Mutations and relevant data given by FireProt. (A) Energy mutants. **(B)** Evolution mutants.
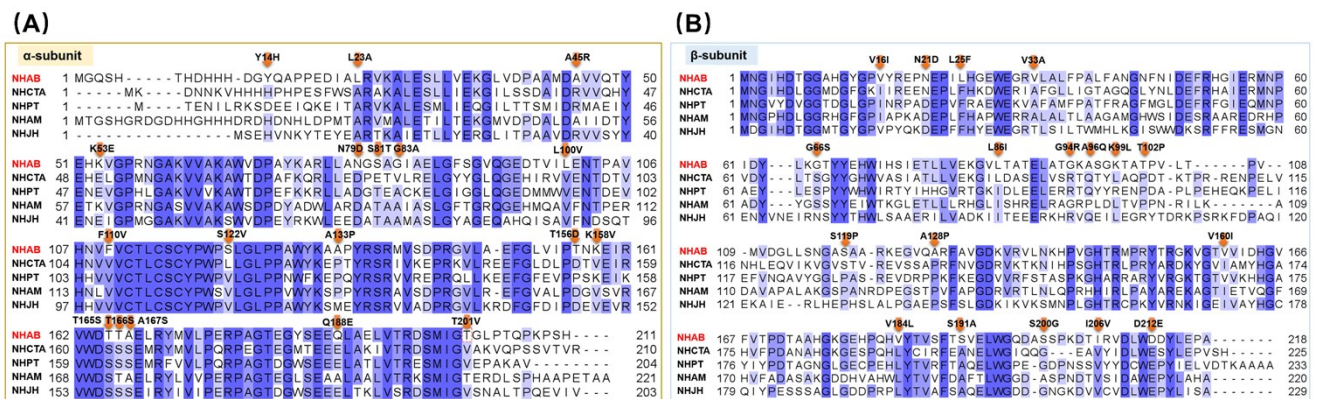
Figure S5. Consensus alignment using Clustal Omega. (A) α-subunit. **(B)** β-subunit.
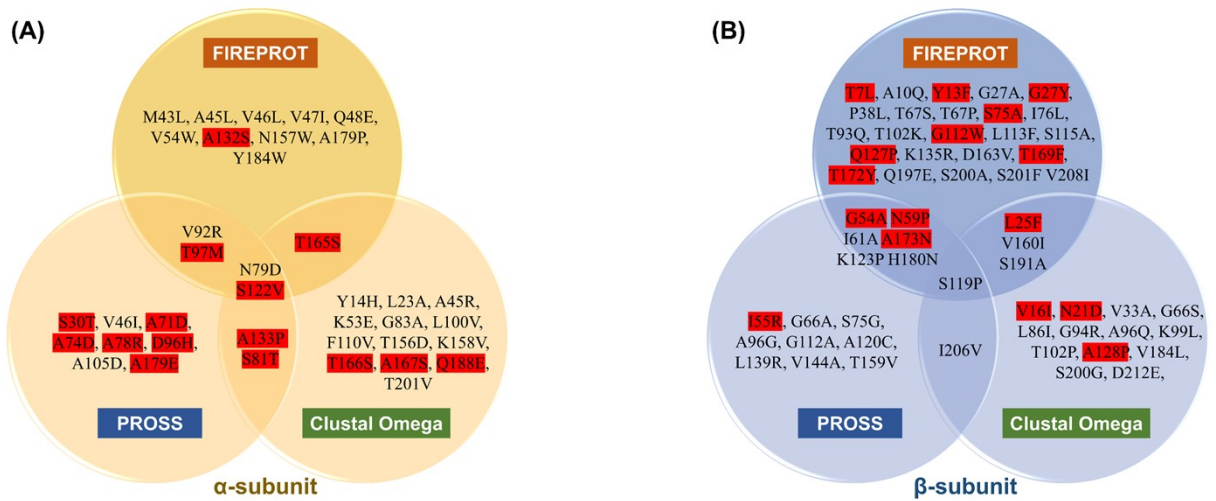
Figure S6. Construction of mutant library. (A) α-subunit. **(B)** β-subunit. Mutations selected for experimental verification were colored in red.

(A)

α-subunit	A45	V46	V47	Q48	N79	G83	A105	T156	K158
α-Cys112	-0.06583	-0.05111	0.00844	0.00405	0.02241	-0.00569	0.04528	0.02050	0.03044
α-CSD115	-0.02677	0.03090	0.05215	-0.00957	0.00155	-0.06699	0.01670	0.03625	0.04322
α-Ser116	-0.02950	-0.00090	0.07239	0.02841	-0.02969	-0.04394	0.02544	0.00064	0.02570
α-CEA117	-0.02806	0.00080	0.03834	0.02843	-0.01259	-0.04257	0.01001	-0.03601	0.00921

β-subunit	G66	T67	L86	T93	A96	L113	S115	A120	L139	V144	T159	V160	V184	S191	Q197	S200	S201
α-Cys112	-0.00612	-0.01217	-0.06066	-0.06129	-0.02646	0.01287	0.0634	0.02678	-0.04049	-0.00769	0.01151	0.04025	-0.01101	0.07317	0.05930	-0.06195	0.07948
α-CSD115	0.06845	0.06684	-0.02212	-0.05468	-0.02924	0.04370	0.01845	-0.05495	-0.0199	0.01728	-0.02159	0.00946	-0.08496	0.05955	0.06093	-0.06255	0.03015
α-Ser116	0.04259	0.03971	-0.05694	-0.05824	-0.05893	0.04122	0.08417	0.01423	-0.07564	0.04088	-0.05355	-0.02956	-0.09885	-0.02697	0.03041	-0.04459	0.00085
α-CEA117	0.08172	0.05264	-0.09024	-0.04500	-0.06110	0.03177	0.07608	0.08414	-0.0992	-0.00111	-0.08466	-0.05459	-0.03058	-0.07429	0.02779	-0.04589	-0.03212

(B)

α-subunit	S30	A71	A74	A78	S81	D96	T97	S122	A132	A133	T165	T166	A167	A179	Q188
α-Cys112	-0.16390	-0.08897	-0.07444	0.00872	0.08160	0.16220	0.13979	0.15741	0.28763	0.36687	0.54627	0.44892	0.28405	-0.11474	-0.10781
α-CSD115	-0.16242	-0.13354	-0.12337	-0.12727	0.01357	0.10586	0.07118	0.25451	0.28844	0.41310	0.22994	0.21975	0.08440	-0.10799	-0.11061
α-Ser116	-0.16912	-0.10439	-0.09800	-0.02834	0.13212	0.14054	0.09856	0.27339	0.40366	0.40758	0.25464	0.23753	0.15347	-0.11743	-0.07369
α-CEA117	-0.17831	-0.08484	-0.09474	-0.11415	0.10780	0.15977	0.11919	0.31778	0.42892	0.32456	0.36769	0.30621	0.23379	-0.12272	-0.04892

β-subunit	T7	Y13	V16	N21	L25	G27	G54	I55	N59	S75	G112	Q127	A128	T169	T172	A173
α-Cys112	0.28954	0.18801	0.15241	0.10294	0.09108	0.06352	0.08667	-0.00668	0.08529	0.10004	-0.10602	-0.10030	-0.10623	0.07844	0.05320	0.11235
α-CSD115	0.37943	0.24788	0.21210	0.13876	0.12893	0.09182	0.20991	0.12732	0.19130	0.19252	-0.14210	-0.14382	-0.16129	0.10262	0.20517	-0.00508
α-Ser116	0.26702	0.15674	0.19556	0.14888	0.17118	0.13625	0.13491	0.11247	0.15554	0.15418	-0.13857	-0.16397	-0.18081	0.09636	0.13651	-0.09854
α-CEA117	0.25377	0.11518	0.17754	0.14245	0.13996	0.10618	0.07194	0.05782	0.09025	0.11366	-0.08592	-0.09738	-0.09852	0.08180	0.12550	-0.11020

Figure S7. cij (dynamics correlation coefficient) with catalytic residue (α-Cys112, α-CSD115, α-Ser116, α-CEA117). (A) cij of mutation site discarded. **(B)** cij of mutation sites selected for experimental determination.
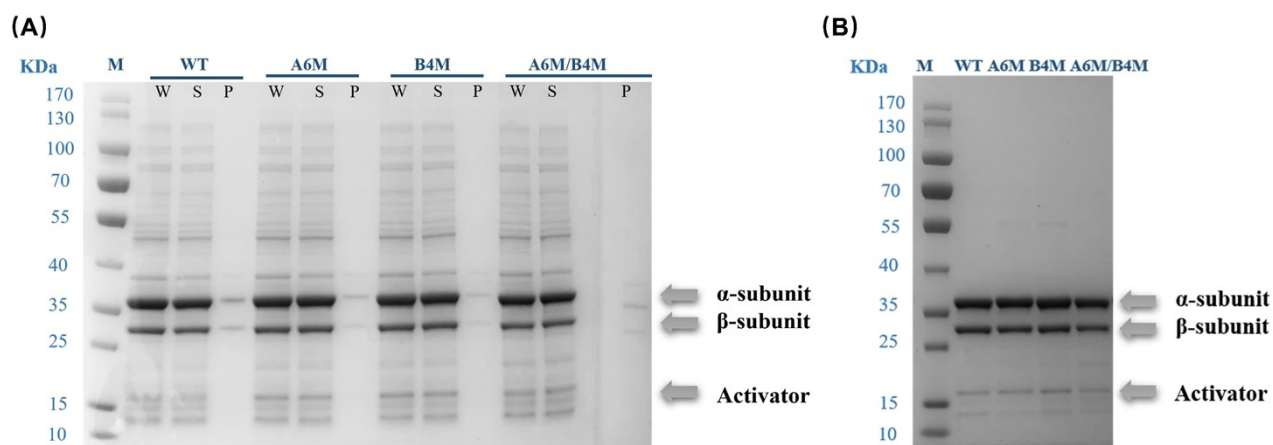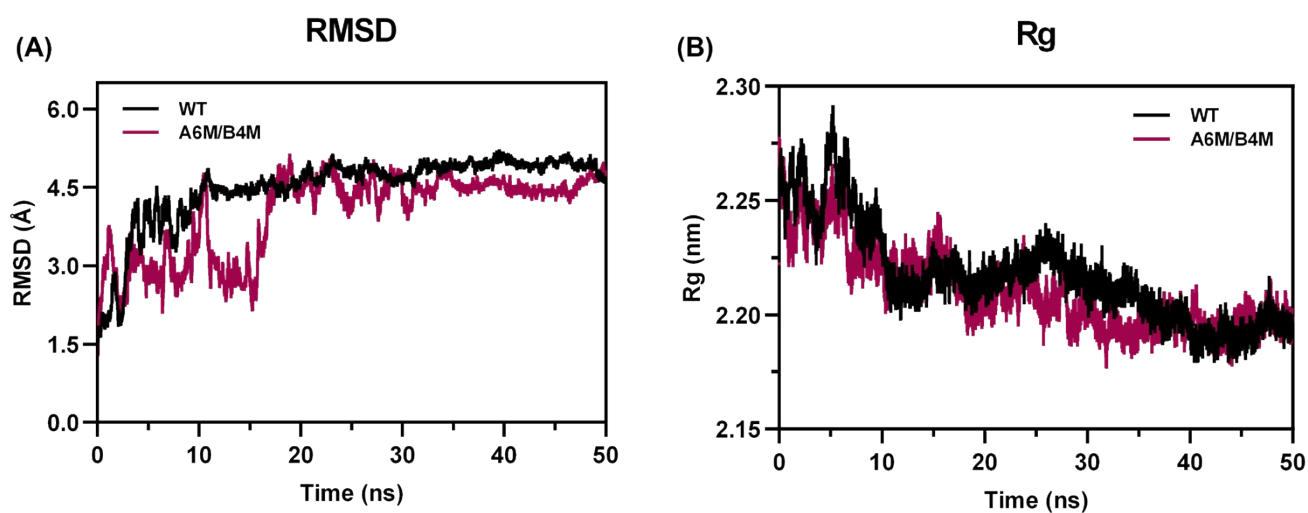
Figure S8. SDS-PAGE analysis of the protein expression of the wild-type NHAB and mutants. (A) Crude extract. M:marker, W: whole cell; S: supernate; P: precipitate. **(B)** Prue enzyme. M:marker, W: whole cell; S: supernate; P: precipitate.
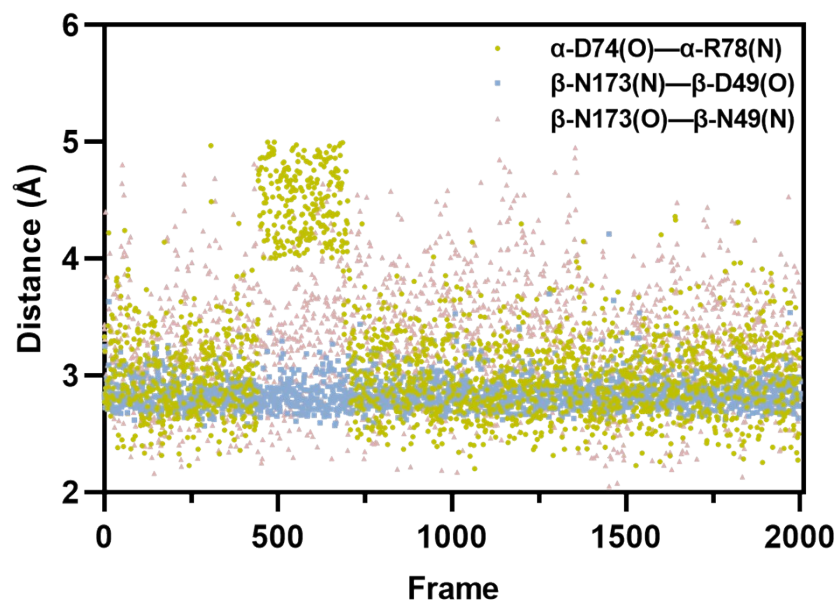
Figure S9. The distance distribution of bonded atoms in A6M/B4M analysed by MD simulation.

Figure S10. (A) RMSD values during the 50 ns simulation. **(B)** Rg values during the 50 ns simulation.
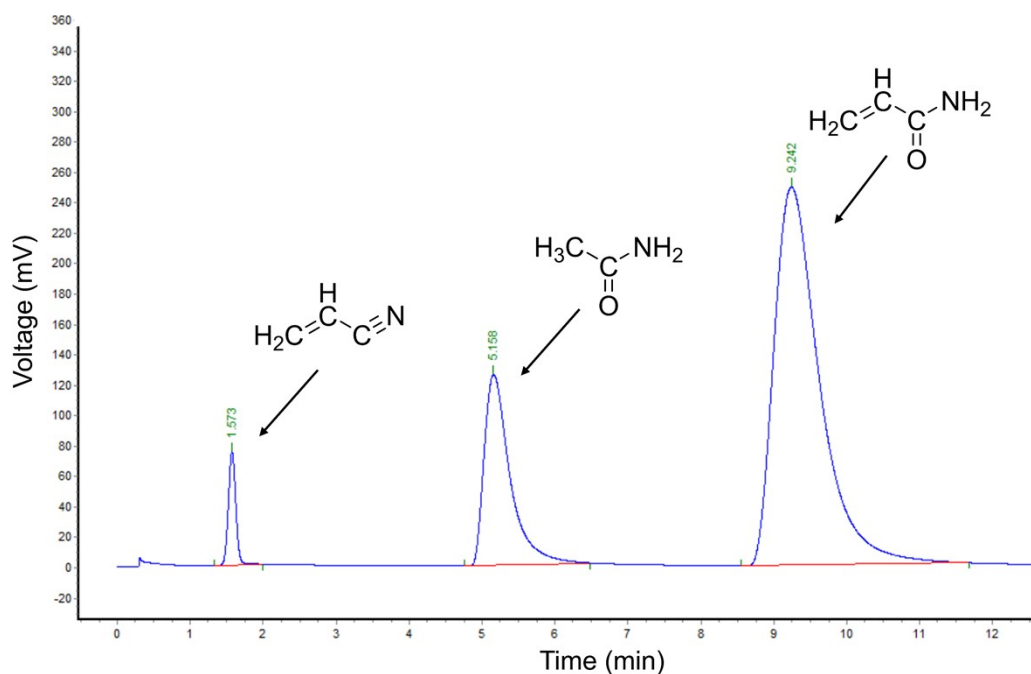
Figure S11. Gas chromatography (GC) analysis of the reactant and product. Acrylonitrile (1.573 min), Acetamide (5.158 min) and Acrylamide (9.242 min).