Supplementary Material

# Supplementary Figures and Tables

## Supplementary Figures



Figure S1. SDS-PAGE of the purification of recombinant pepsin and mutations with improved thermostability. Lane M. standard, 1. wild-type, 2. A24P, 3. D52N, 4. Q55R, 5. S129A.



Figure S2. The RMSD of protein backbone at the WT, D52N and S129A three systems during 100 ns simulation.



Figure S3. The Michaelis−Menten plots for the kinetic investigations of the wild-type pepsin, mutants D52N and S129A.

## Supplementary Tables

Table S1. Primers for site-directed mutagenesis.

|  |  |
| --- | --- |
| Primer | Sequence (5’-3’) |
| Primer-F | GCTGAAGCTTACGTAGAATTCCTCGTCAAGGTCCCG |
| Primer-R | TAAGGCGAATTAATTCGCGGCCGCTTTTTTTCAGGAGGT |
| D3E-F | GCCGCTGCCCTGATAGGCGAGGAGCCCCTTGAGAACTA |
| D3E-R | TAGTTCTCAAGGGGCTCCTCGCCTATCAGGGCAGCGGC |
| E4I-F | GCTGCCCTGATAGGCGATGTTCCCCTTGAGAACTACCT |
| E4I-R | AGGTAGTTCTCAAGGGGAACATCGCCTATCAGGGCAGC |
| E4L-F | GCTGCCCTGATAGGCGATCTGCCCCTTGAGAACTACCTG |
| E4L-R | CAGGTAGTTCTCAAGGGGCAGATCGCCTATCAGGGCAGC |
| E4V-F | GCTGCCCTGATAGGCGATGTTCCCCTTGAGAACTACCTG |
| E4V-R | CAGGTAGTTCTCAAGGGGAACATCGCCTATCAGGGCAGC |
| A24P-F | ATCGGCATTGGAACCCCCCCGCAGGACTTCACCGTCATC |
| A24P-R | GATGACGGTGAAGTCCTGCGGGGGGGTTCCAATGCCGAT |
| T28Y-F | ACCCCCGCTCAGGACTTCTATGTCATCTTTGACACCGGC |
| T28Y-R | GCCGGTGTCAAAGATGACATAGAAGTCCTGAGCGGGGGT |
| D52N-F | TCTAGTCTCGCCTGCAGCAATCACAACCAGTTCAACCCT |
| D52N-R | AGGGTTGAACTGGTTGTGATTGCTGCAGGCGAGACTAGA |
| Q55R-F | GCCTGCAGCGACCACAACCGTTTCAACCCTGATGACTCC |
| Q55R-R | GGAGTCATCAGGGTTGAAACGGTTGTGGTCGCTGCAGGC |
| E65Q-F | GATGACTCCTCCACCTTCCAGGCCACCAGCCAGGAGCTG |
| E65Q-R | CAGCTCCTGGCTGGTGGCCTGGAAGGTGGAGGAGTCATC |
| A66L-F | GACTCCTCCACCTTCGAGCTGACCAGCCAGGAGCTGTCC |
| A66L-R | GGACAGCTCCTGGCTGGTCAGCTCGAAGGTGGAGGAGTC |
| G85I-F | AGCATGACAGGCATCCTTATTTACGACACCGTCCAGGTC |
| G85I-R | GACCTGGACGGTGTCGTAAATAAGGATGCCTGTCATGCT |
| G85V-F | AGCATGACAGGCATCCTTGTTTACGACACCGTCCAGGTC |
| G85V-R | GACCTGGACGGTGTCGTAAACAAGGATGCCTGTCATGCT |
| D87V-F | ACAGGCATCCTTGGATACGTTACCGTCCAGGTCGGAGGC |
| D87V-R | GCCTCCGACCTGGACGGTAACGTATCCAAGGATGCCTGT |
| D87Y-F | ACAGGCATCCTTGGATACTATACCGTCCAGGTCGGAGGC |
| D87Y-R | GCCTCCGACCTGGACGGTATAGTATCCAAGGATGCCTGT |
| D96L-F | CAGGTCGGAGGCATCAGCCTGACCAACCAGATCTTTGGC |
| D96L-R | GCCAAAGATCTGGTTGGTCAGGCTGATGCCTCCGACCTG |
| G102F-F | GACACCAACCAGATCTTTTTCCTGAGCGAGACAGAG |
| G102F-R | CTCTGTCTCGCTCAGGAAAAAGATCTGGTTGGTGTC |
| G102Y-F | GACACCAACCAGATCTTTTATCTGAGCGAGACAGAG |
| G102Y-R | CTCTGTCTCGCTCAGATAAAAGATCTGGTTGGTGT |
| G102W-F | GACACCAACCAGATCTTTTGGCTGAGCGAGACAGAG |
| G102W-R | CTCTGTCTCGCTCAGCCAAAAGATCTGGTTGGTGTC |
| Y114R-F | GGCTCCTTCCTGTACCGTGCCCCCTTCGACGGC |
| Y114R-R | GCCGTCGAAGGGGGCACGGTACAGGAAGGAGCC |
| S129A-F | CTGGCCTACCCCAGCATCGCAGCCTCCGGGGCCACCCCT |
| S129A-R | AGGGGTGGCCCCGGAGGCTGCGATGCTGGGGTAGGCCAG |
| L140M-F | ACCCCTGTCTTTGACAACATGTGGGACCAGGGCCTGGTT |
| L140M-R | AACCAGGCCCTGGTCCCACATGTTGTCAAAGACAGGGGT |
| W141M-F | CCTGTCTTTGACAACCTGATGGACCAGGGCCTGGTTTCC |
| W141M-R | GGAAACCAGGCCCTGGTCCATCAGGTTGTCAAAGACAGG |
| D159G-F | GTCTACCTGAGCTCCAATGGTGATAGCGGCAGTGTGGTG |
| D159G-R | CACCACACTGCCGCTATCACCATTGGAGCTCAGGTAGAC |
| L167F-F | AGCGGCAGTGTGGTGTTGTTTGGCGGCATCGATTCTTCT |
| L167F-R | AGAAGAATCGATGCCGCCAAACAACACCACACTGCCGCT |
| D200N-F | TTGGACAGCATCACCATGAATGGAGAGACCATCGCTTGC |
| D200N-R | GCAAGCGATGGTCTCTCCATTCATGGTGATGCTGTCCAA |
| T222V-F | ACGGGCACCTCTCTGCTGGTTGGCCCCACCAGTGCCATT |
| T222V-R | AATGGCACTGGTGGGGCCAACCAGCAGAGAGGTGCCCGT |
| Q232W-F | AGTGCCATTGCCAACATCTGGAGCGACATCGGAGCCAGC |
| Q232W-R | GCTGGCTCCGATGTCGCTCCAGATGTTGGCAATGGCACT |
| T261V-F | CTGCCTGACATCGTCTTCGTTATCAACGGTGTCCAGTAC |
| T261V-R | GTACTGGACACCGTTGATAACGAAGACGATGTCAGGCAG |
| S270P-F | GGTGTCCAGTACCCTCTGCCGCCCAGTGCCTACATCCTG |
| S270P-R | CAGGATGTAGGCACTGGGCGGCAGAGGGTACTGGACACC |
| T283F-F | CAGGACGATGACAGCTGCTTTAGCGGCTTCGAGGGCATG |
| T283F-R | CATGCCCTCGAAGCCGCTAAAGCAGCTGTCATCGTCCTG |
| Q308L-F | GGTGACGTCTTCATCCGCCTGTACTACACCGTCTTTGAC |
| Q308L-R | GTCAAAGACGGTGTAGTACAGGCGGATGAAGACGTCACC |
| R315F-F | TACTACACCGTCTTTGACTTTGCCAACAACAAGGTCGGC |
| R315F-R | GCCGACCTTGTTGTTGGCAAAGTCAAAGACGGTGTAGTA |
| R315W-F | TACTACACCGTCTTTGACTGGGCCAACAACAAGGTCGGC |
| R315W-R | GCCGACCTTGTTGTTGGCCCAGTCAAAGACGGTGTAGTA |
| K319F-F | GTCTTTGACAGGGCCAACAACTTTGTCGGCCTGGCTCCCGTG |
| K319F-R | CACGGGAGCCAGGCCGACAAAGTTGTTGGCCCTGTCAAAGAC |

Table S2. Prediction results of the top twenty mutants by PoPMuSiC.

|  |  |  |  |
| --- | --- | --- | --- |
| Residue Number | Wild-type | Mutant | $$∆∆G (kcal/mol)$$ |
| 87 | ASP | TYR | -1.84 |
| 87 | ASP | PHE | -1.8 |
| 87 | ASP | ILE | -1.71 |
| 4 | GLU | ILE | -1.71 |
| 87 | ASP | VAL | -1.59 |
| 4 | GLU | VAL | -1.59 |
| 4 | GLU | PHE | -1.59 |
| 4 | GLU | LEU | -1.5 |
| 87 | ASP | LEU | -1.43 |
| 87 | ASP | MET | -1.43 |
| 87 | ASP | TRP | -1.42 |
| 4 | GLU | MET | -1.42 |
| 102 | GLY | TRP | -1.33 |
| 223 | GLY | ILE | -1.25 |
| 87 | ASP | CYS | -1.24 |
| 223 | GLY | LEU | -1.24 |
| 159 | ASP | GLY | -1.22 |
| 4 | GLU | TYR | -1.21 |
| 102 | GLY | PHE | -1.19 |
| 102 | GLY | TYR | -1.16 |

Table S3. Prediction results of the top twenty mutants by HoTMuSiC.

|  |  |  |  |
| --- | --- | --- | --- |
| Residue Number | Wild-type | Mutant | $$∆T\_{m} (°C)$$ |
| 4 | GLU | ILE | 4.11 |
| 4 | GLU | VAL | 3.77 |
| 4 | GLU | PHE | 3.72 |
| 102 | GLY | TRP | 3.72 |
| 102 | GLY | PHE | 3.55 |
| 76 | GLY | TRP | 3.41 |
| 87 | ASP | VAL | 3.37 |
| 102 | GLY | TYR | 3.34 |
| 87 | ASP | PHE | 3.26 |
| 4 | GLU | LEU | 3.17 |
| 4 | GLU | TYR | 3.09 |
| 87 | ASP | TYR | 3.05 |
| 4 | GLU | MET | 2.97 |
| 87 | ASP | ILE | 2.96 |
| 76 | GLY | PHE | 2.71 |
| 87 | ASP | TRP | 2.7 |
| 76 | GLY | TYR | 2.68 |
| 46 | SER | TRP | 2.56 |
| 87 | ASP | LEU | 2.48 |
| 294 | SER | TRP | 2.42 |

Table S4. Prediction results of the top twenty mutants by ETSS.

|  |  |  |
| --- | --- | --- |
| Residue Number | Wild-type | $$∆G\_{qq} (kcal/mol)$$ |
| 310 | TYR | 6.77 |
| 267 | TYR | 6.72 |
| 309 | TYR | 6.57 |
| 14 | TYR | 6.27 |
| 307 | ARG | 6.18 |
| 75 | TYR | 6.17 |
| 125 | TYR | 6.17 |
| 175 | TYR | 6.07 |
| 154 | TYR | 5.85 |
| 274 | TYR | 5.64 |
| 315 | ARG | 5.55 |
| 174 | TYR | 5.49 |
| 114 | TYR | 5.44 |
| 53 | HIS | 5.44 |
| 189 | TYR | 5.18 |
| 113 | TYR | 5.02 |
| 9 | TYR | 4.92 |
| 319 | LYS | 4.90 |
| 86 | TYR | 4.42 |
| 44 | TYR | 4.23 |

Table S5. Prediction results of the top twenty mutants by DeepDDG.

|  |  |  |  |
| --- | --- | --- | --- |
| Residue Number | Wild-type | Mutant | $$∆∆G (kcal/mol)$$ |
| 96 | ASP | LEU | 5.497 |
| 96 | ASP | ILE | 4.9 |
| 96 | ASP | PHE | 4.43 |
| 96 | ASP | TRP | 4.303 |
| 96 | ASP | VAL | 4.132 |
| 96 | ASP | TYR | 3.948 |
| 96 | ASP | MET | 3.512 |
| 96 | ASP | THR | 3.083 |
| 96 | ASP | CYS | 2.821 |
| 85 | GLY | LEU | 2.501 |
| 85 | GLY | PHE | 2.464 |
| 85 | GLY | TRP | 2.461 |
| 85 | GLY | ILE | 2.294 |
| 85 | GLY | TYR | 2.12 |
| 205 | ALA | TRP | 2.01 |
| 227 | ALA | LEU | 1.948 |
| 102 | GLY | PHE | 1.875 |
| 102 | GLY | LEU | 1.772 |
| 85 | GLY | VAL | 1.751 |
| 96 | ASP | HIS | 1.743 |