**Bifidobacterial GH146 β-L-arabinofuranosidase for the removal of β1,3-L-arabinofuranosides on plant glycans**

Kiyotaka Fujita[1,2]*, Hanako Tsunomachi[1], Pan Lixia[3,4], Shun Maruyama[3], Masayuki Miyake[3], Aimi Dakeshita[1], Kanefumi Kitahara[1,2], Katsunori Tanaka[5,6], Yukishige Ito[5,7], Akihiro Ishiwata[5], Shinya Fushinobu[3,8]*

1. Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan

2. The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan

3. Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

4. The National Engineering Research Center for Non-Food Biorefinery, State Key Laboratory of Non-Food Biomass and Enzyme Technology, Guangxi Academy of Sciences, Nanning 530007, Guangxi, China

5. RIKEN, Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

6. Department of Chemical Science and Engineering, Tokyo Institute of Technology
2-12-1 Ookayama, Meguro-ku, Tokyo 152-8552, Japan

7. Graduate School of Science, Osaka University
1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043 Japan

8. Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan

* **Address correspondence to:** Kiyotaka Fujita, k4022897@kadai.jp; Shinya Fushinobu, asfushi@mail.ecc.u-tokyo.ac.jp

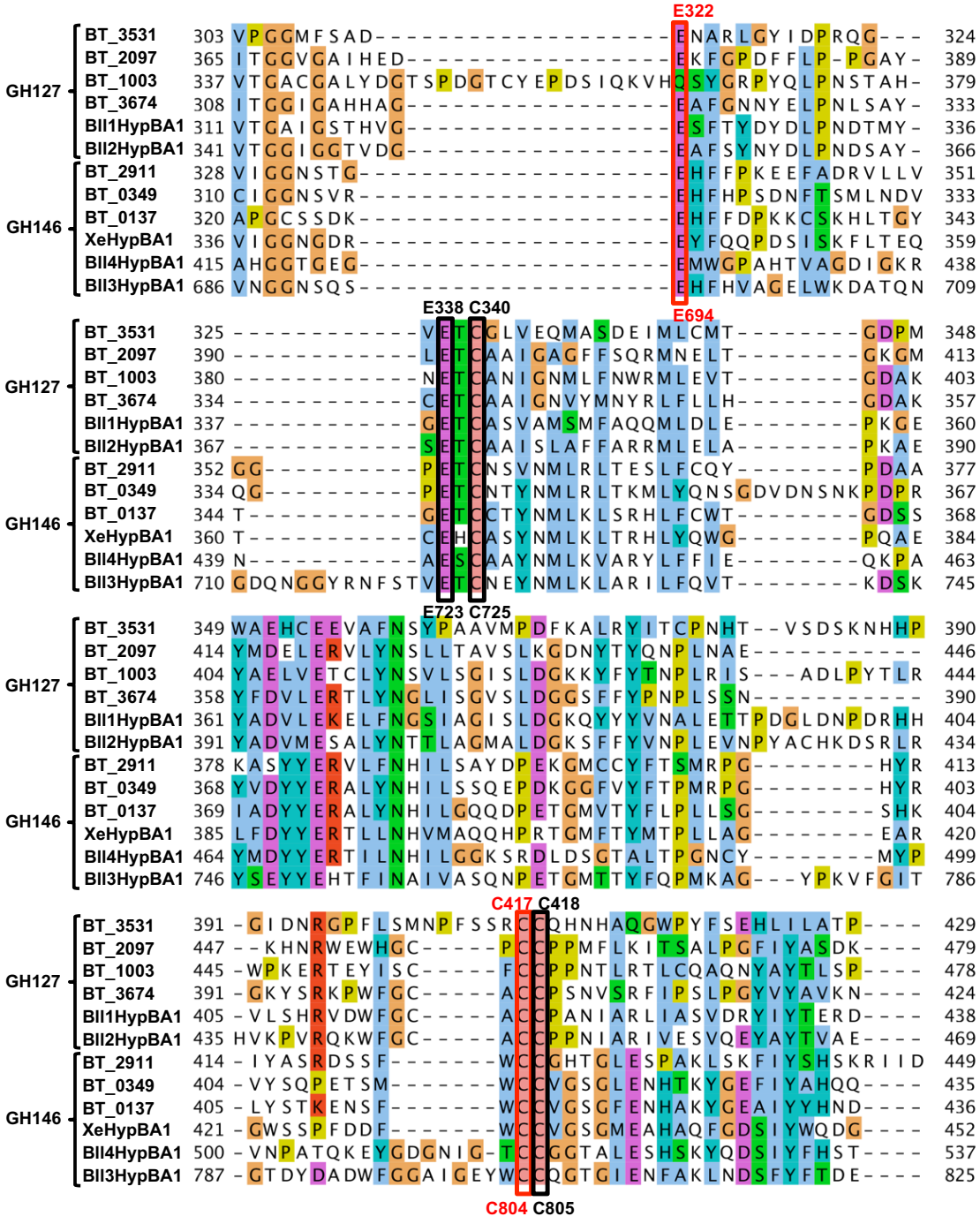


**Figure S1.** Amino acid sequence alignment of Bll3HypBA1 and GH127/146 β-L-arabinofuranosidases. The catalytic residues (nucleophile and acid/base catalyst) are boxed with magenta lines. The Zn-coordinating residues are boxed with black lines. The amino acid residues for Bll1HypBA1 and Bll3HypBA1 are shown above and below the box, respectively.

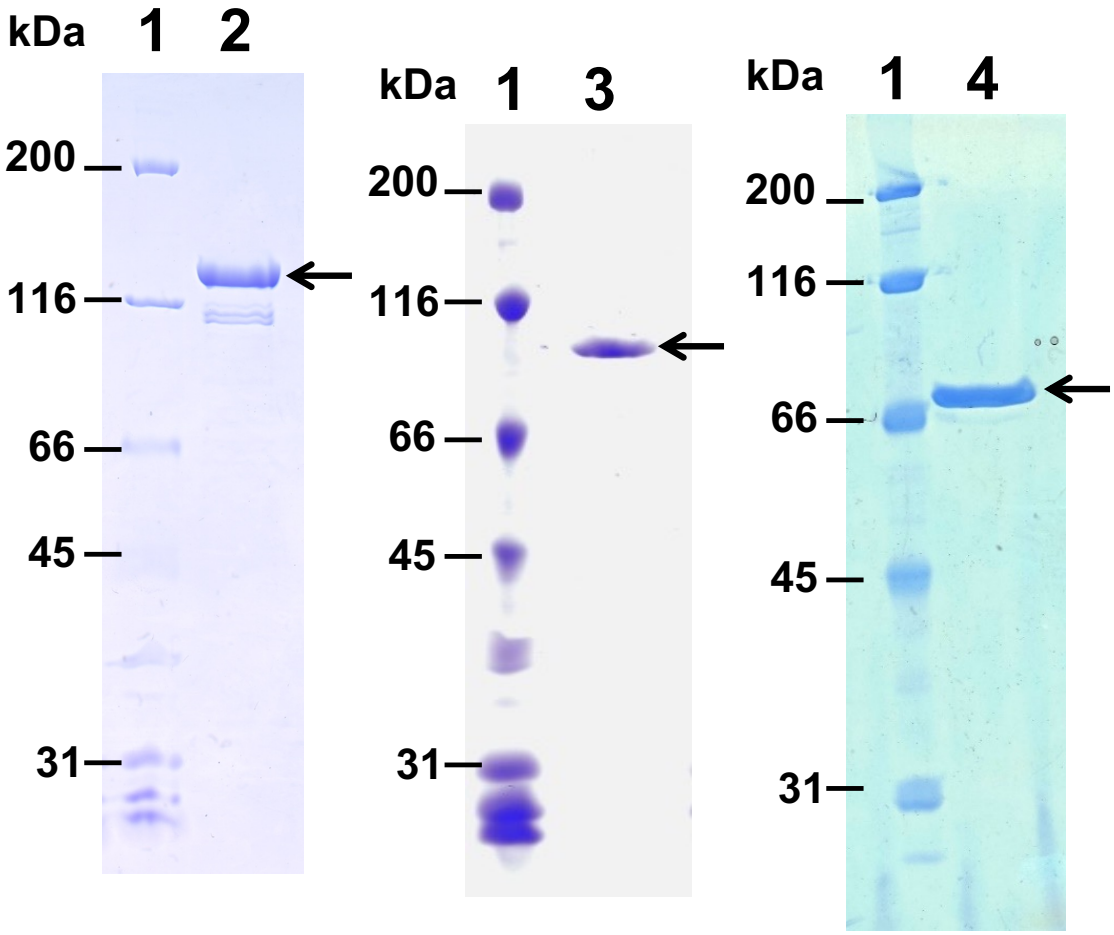


**Figure S2.** SDS-PAGE analysis of recombinant Bll3HypBA1 proteins. Purified proteins were electrophoresed on a 5–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular size marker; lane 2, Bll3HypBA1-NΔ35CΔ761; lane 3, Bll3HypBA1-NΔ379CΔ761; lane 4, NΔ379CΔ933 (lane 4). Arrows indicate target proteins at expected molecular size.

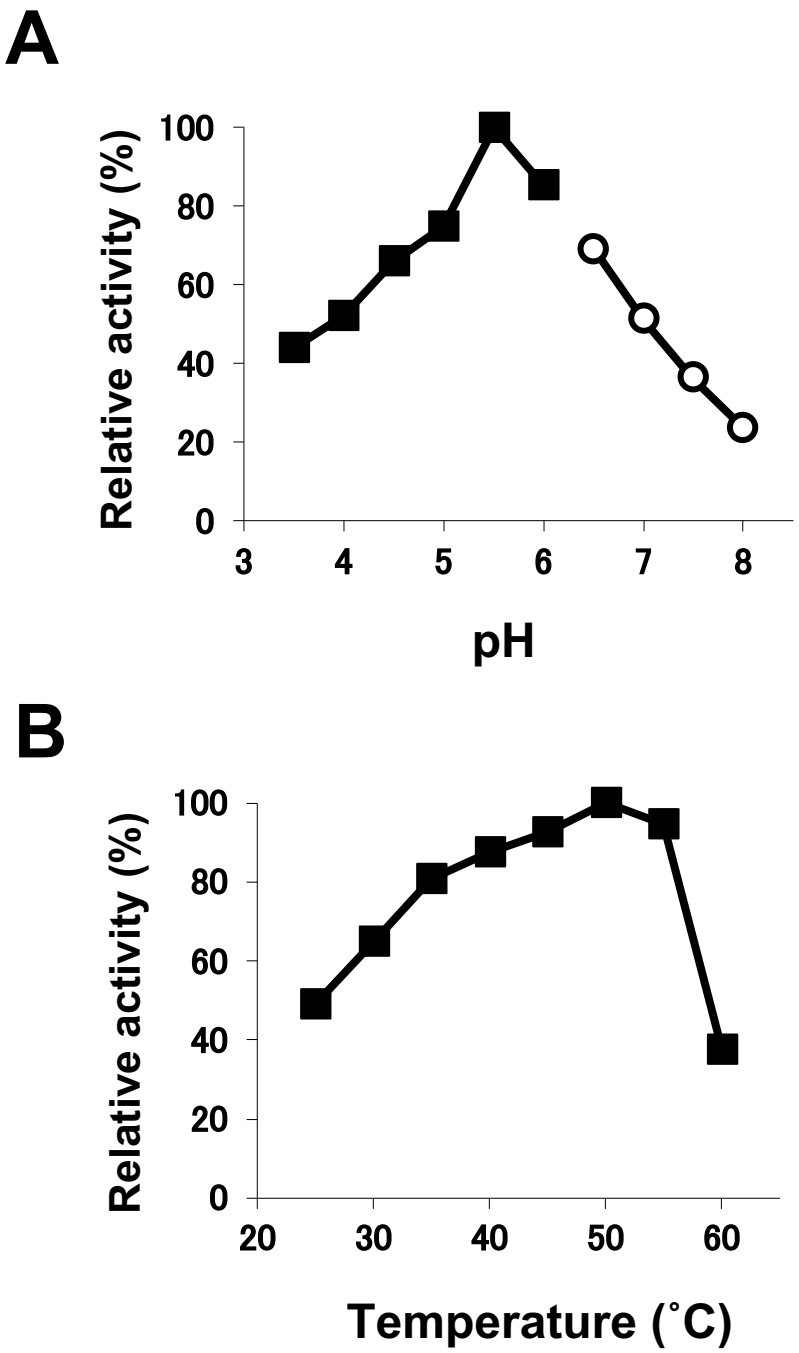


**Figure S3.** Optimal pH and temperature of Bll3HypBA1-NΔ35CΔ761. (**A**) pH dependence of the activity in various buffers at 40 °C for 20 min. Sodium acetate buffer (closed squares) and sodium phosphate buffer (open circles) were used. Enzyme activities are expressed as a percentage of the activity in sodium acetate buffer at pH 5.5. (**B**) The temperature dependence of the activity at pH 5.5 for 20 min. The enzymatic activities are expressed as the percentage of the activity at 50 °C.

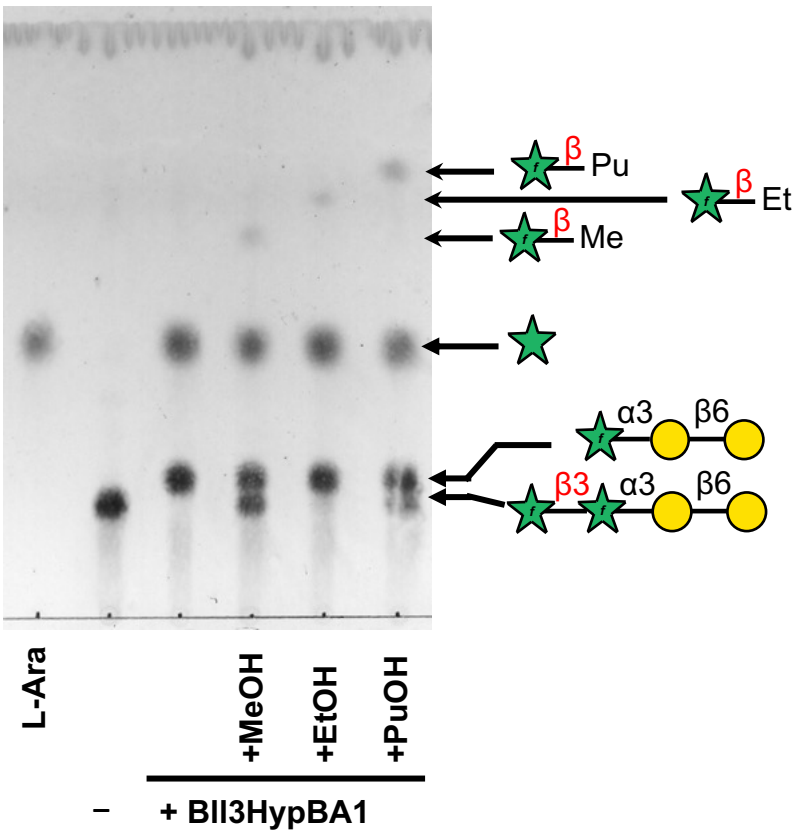


**Figure S4.** Transglycosylation activity of Bll3HypBA1 in the presence of 1-alkanols. Thin-layer chromatography analysis of reaction products. Bll3HypBA1-NΔ35CΔ761was incubated with L-Ara*f*-β1,3-L-Ara*f*-α1,3-Gal-β1,6-Gal in the presence of 5% methanol, ethanol, or 1-propanol.

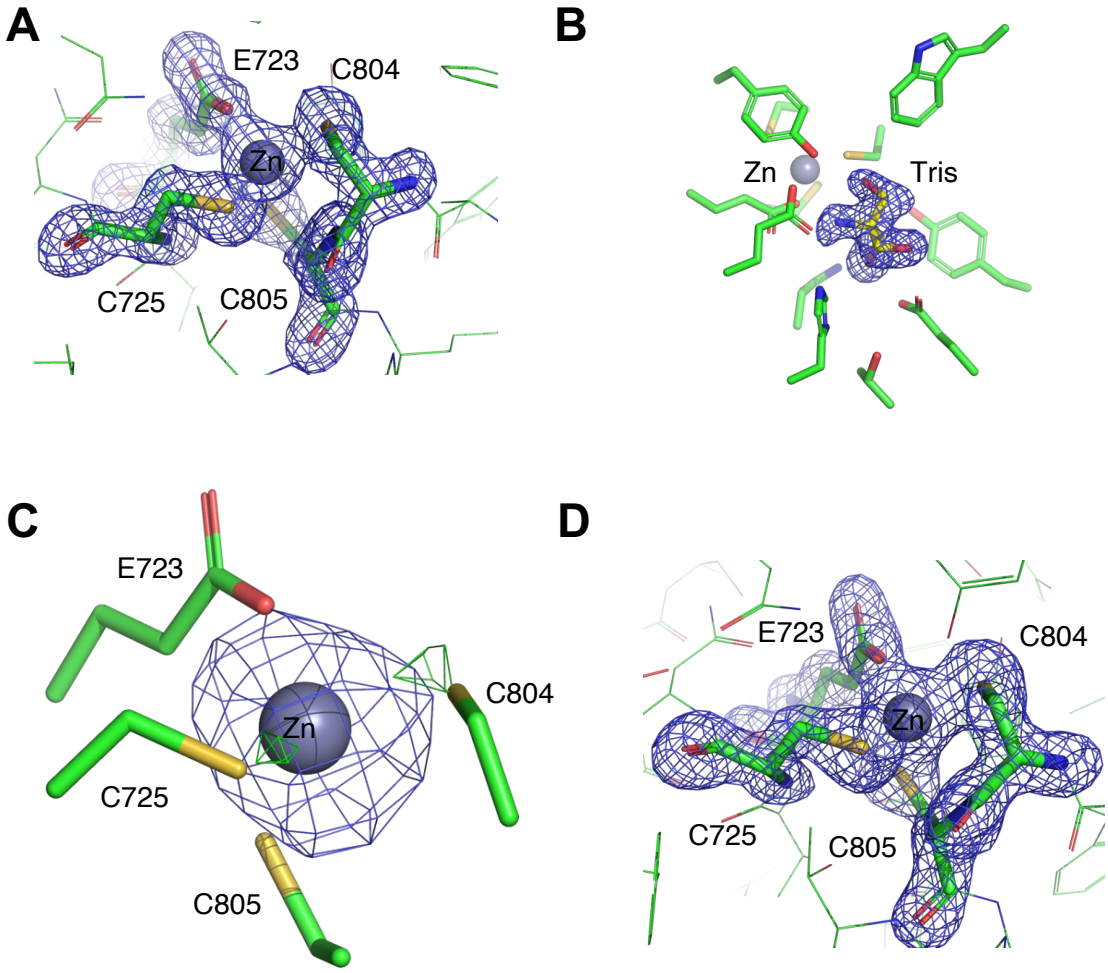


**Figure S5.** Electron density maps.

(**A**) Zn and coordinating residues in NΔ379CΔ761 with a polder map (3σ). (**B**) Tris molecule bound to the active site of NΔ379CΔ761 with a polder map (4σ). (**C**) Anomalous difference maps of the Zn atom. Data collected at 1.280 Å (5σ, blue) and 1.3000 Å (3.5σ, green) are shown. (**D**) Zn and coordinating residues in NΔ379CΔ933 with a polder map (3σ).

**Table S1.** The primers for the deletion and site-directed mutagenesis.

| Name | Sequence of oligonucleotide primers |
|---|---|
| NΔ_Reverse_Primer | 5′- CATGGTATATCTCCTTCTTAAAGTT -3′ |
| NΔ379rev | 5'- GAGAACGTCACCGTAGCCGACGAATA -3' |
| CΔ_Forward_Primer | 5′-CTCGAGCACCACCACCACCACTG-3′ |
| CΔ933rev | 5'- GACCTCGGCCATATCCCAGTAGATC -3' |
| W429A mutation _for | 5′- GGCGGTGCGGAGAACGGCCCGGACGAG-3′ |
| W429A mutation _rev | 5′- GTTCTCCGCACCGCCGTAGTTCTTCGC-3′ |
| Y526A mutation _for | 5′- CGCTTCGCGAACCTGCACAAGGTTGAG-3′ |
| Y526A mutation _rev | 5′- CAGGTTCGCGAACGGCACGATCAGGCC-3′ |
| E578A mutation _for | 5′- CGCACTGCGTACGGCGGCATGAATGAC-3′ |
| E578A mutation _rev | 5′- GCCGTACGCAGTGCGCAGCATGTCGGT-3′ |
| H628A mutation _for | 5′- GGCTTGGCGGCCAACACCACGATTCCG-3′ |
| H628A mutation _rev | 5′- GTTGGCCGCCAAGCCGTTGAGCGGATC-3′ |
| N630A mutation _for | 5′- CACGCCGCGACCACGATTCCGAAGCTC-3′ |
| N630A mutation _rev | 5′- CGTGGTCGCGGCGTGCAAGCCGTTGAG-3′ |
| T631A mutation _for | 5′- GCCAACGCGACGATTCCGAAGCTCACC-3′ |
| T631A mutation _rev | 5′- AATCGTCGCGTTGGCGTGCAAGCCGTT-3′ |
| E694Q mutation _for | 5′- CAGTCCCAGCACTTCCACGTGGCCGGT -3′ |
| E694Q mutation _rev | 5′- GAAGTGCTGGGACTGCGAGTTGCCGCC -3′ |
| E694A mutation _for | 5′- CAGTCCGCGCACTTCCACGTGGCCGGT -3′ |
| E694A mutation _rev | 5′- GAAGTGCGCGGACTGCGAGTTGCCGCC -3′ |
| E723Q mutation _for | 5′- ACCGTGCAGACCTGCAACGAGTACAAC -3′ |
| E723Q mutation _rev | 5′- GCAGGTCTGCACGGTGGAGAAGTTGCG -3′ |
| E723A mutation _for | 5′- ACCGTGGCGACCTGCAACGAGTACAAC -3′ |
| E723A mutation _rev | 5′- GCAGGTCGCCACGGTGGAGAAGTTGCG -3′ |
| C725S mutation _for | 5′- GAGACCAGCAACGAGTACAACATGCTC -3′ |
| C725S mutation _rev | 5′- CTCGTTGCTGGTCTCCACGGTGGAGAA -3′ |
| C725A mutation _for | 5′- GAGACCGCGAACGAGTACAACATGCTC -3′ |
| C725A mutation _rev | 5′- CTCGTTCGCGGTCTCCACGGTGGAGAA -3′ |
| C804S mutation _for | 5′- TATTGGAGCTGCCAGGGTACCGGTATC -3′ |
| C804S mutation _rev | 5′- CTGGCAGCTCCAATACTCGCCAATCGC -3′ |

| | |
|---|---|
| C805S mutation _for | 5′- TGGTGCAGCCAGGGTACCGGTATCGAG -3′ |
| C805S mutation _rev | 5′- ACCCTGGCTGCACCAATACTCGCCAAT -3′ |
| C805A mutation _for | 5′- TGGTGCGCGCAGGGTACCGGTATCGAG -3′ |
| C805A mutation _rev | 5′- ACCCTGCGCGCACCAATACTCGCCAAT -3′ |

The positions of the mutated sequences are underlined.

**Table S2.** Comparison of the specific activities of Bll3HypBA1-NΔ35CΔ761 and Bll3HypBA1-NΔ379CΔ761 towards oligosaccharide and polysaccharide.

| Substrates | NΔ35CΔ761 (Units/μmol)[a] | NΔ379CΔ761 (Units/μmol)[a] | Fold[b] |
|---|---|---|---|
| Ara*f*-β1,3-Ara*f*$Gal_2$-ABEE | 505 | 860 | 0.59 |
| larch AGP | 29.0 | 4.99 | 5.8 |
| arabinan | 515 | 309 | 1.7 |
| gum arabic | 95.7 | 86.8 | 1.1 |

[a]Specific activity was calculated with the calculated molecular mass of 129,964 Da for Bll3HypBA1-NΔ35CΔ761 and 94,145 Da for Bll3HypBA1-NΔ379CΔ761.

[b]Ratio of the specific activities of Bll3HypBA1-NΔ35CΔ761 to Bll3HypBA1-NΔ379CΔ761.

**Table S3.** X-ray crystallographic data collection and refinement statistics of Bll3HypBA1.

| Data set | NΔ379CΔ761 SeMet | NΔ379CΔ761 + Tris | NΔ379CΔ761 Zn peak | NΔ379CΔ761 Zn low remote | NΔ379CΔ933 Ligand free |
|---|---|---|---|---|---|
| Data collection[a] | | | | | |
| Beamline | PF-AR NE3A | PF-AR NE3A | PF-AR NW12A | PF-AR NW12A | PF BL17A |
| Wavelength (Å) | 0.9791 | 1.0000 | 1.2800 | 1.3000 | 0.9800 |
| Space group | $P2_1$ | $P2_12_12_1$ | $P2_1$ | $P2_1$ | $P2_1$ |
| Unit cell (Å, °) | $a$ = 59.612, $b$ = 112.325, $c$ = 102.747, $\beta$ = 96.306 | $a$ = 59.592, $b$ = 111.571, $c$ = 153.010 | $a$ = 59.582, $b$ = 111.698, $c$ = 113.771, $\beta$ = 104.776 | $a$ = 59.588, $b$ = 111.708, $c$ = 113.778, $\beta$ = 104.781 | $a$ = 75.231, $b$ = 83.510, $c$ = 102.034, $\beta$ = 90.659 |
| Resolution (Å) | 49.21–2.00 | 49.71–1.75 | 49.35–2.80 | 49.35–2.80 | 49.20–1.70 |
| $R_{merge}$ | 0.141 (0.533) | 0.176 (1.014) | 0.175 (0.445) | 0.174 (0.442) | 0.076 (0.939) |
| $R_{pim}$ | 0.032 (0.132) | 0.102 (0.592) | 0.104 (0.264) | 0.103 (0.262) | 0.048 (0.595) |
| Total reflections | 1847886 (74746) | 695814 (34242) | 136143 (17948) | 136125 (17942) | 469771 (22932) |
| Unique reflections | 90717 (4451) | 103620 (5038) | 35591 (4702) | 35597 (4702) | 137533 (6721) |
| Mean $I/\sigma(I)$ | 20.1 (6.0) | 8.9 (2.1) | 5.0 (2.3) | 5.0 (2.3) | 7.3 (1.0) |
| $CC_{1/2}$ | 0.998 (0.963) | 0.993 (0.696) | 0.976 (0.872) | 0.976 (0.871) | 0.998 (0.676) |
| Completeness (%) | 100.0 (100.0) | 100.0 (100.0) | 100.0 (100.0) | 100.0 (100.0) | 99.1 (98.3) |
| Multiplicity | 20.4 (16.8) | 6.7 (6.8) | 3.8 (3.8) | 3.8 (3.8) | 3.4 (3.4) |
| Anomalous | 99.7 (99.2) | – | 94.4 (91.9) | 94.3 (91.8) | – |

| | | | | | |
|---|---|---|---|---|---|
| completeness (%) | | | | | |
| Anomalous multiplicity | 10.2 (8.4) | – | 1.9 (2.0) | 1.9 (2.0) | – |
| Wilson B ($Å^2$) | 11.52 | 5.03 | 13.22 | 14.03 | 18.21 |
| Mol/ASU[b] | 1 | 1 | 1 | 1 | 2 |
| Refinement | | | | | |
| Resolution (Å) | | 49.76–1.75 | | | 43.57–1.70 |
| No. of reflections | | 103529 | | | 137521 |
| $R_{work}/R_{free}$ | | 0.1429/0.1673 | | | 0.2202/0.2594 |
| Number of atoms | | 6216 | | | 11092 |
| Residues | | M379–V1051 | | | M379–E1050 (A/B) |
| MolProbity score | | 1.09 | | | 2.16 |
| Clashscore | | 1.65 | | | 1.21 |
| RMSD from ideal values | | | | | |
| Bond lengths (Å) | | 0.0118 | | | 0.0079 |
| Bond angles (°) | | 1.64 | | | 1.43 |
| Ramachandran plot (%) | | | | | |
| Favored | | 97.02 | | | 96.64 |
| Allowed | | 2.98 | | | 3.21 |
| Outlier | | 0.00 | | | 0.15 |
| PDB code | – | 8K7X | – | – | 8K7Y |

[a] Values in parentheses are for the highest resolution shell.

[b] Number of molecules per asymmetric unit.

**Table S4.** Specific activities of Bll3HypBA1 mutants.

| Mutants | Specific activity (Units/mg) | Relative activity[a] (%) |
|---|---|---|
| Wild type | 3.33 | 100 |
| E694Q | $3.63\times10^{-4}$ | 0.01 |
| E694A | $1.43\times10^{-4}$ | < 0.01 |
| E723Q | $5.49\times10^{-3}$ | 0.17 |
| E723A | $6.10\times10^{-5}$ | < 0.01 |
| C725S | $3.35\times10^{-1}$ | 10 |
| C804S | $2.12\times10^{-4}$ | < 0.01 |
| C805S | $1.71\times10^{-4}$ | < 0.01 |
| E694Q/C804S | ND | ND |

[a]Relative activity was expressed as the percentage of the activity against the wild type Bll3HypBA1-NΔ379CΔ933 enzyme.