Bifidobacterial GH146 β -L-arabinofuranosidase for the removal of β 1,3-L-arabinofuranosides on plant glycans

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	BII1HypBA1	337							G	T	CA S	S V	AM	<mark>S</mark> №	1 F /	AQ	QN	ILC	L	E –				· - F	K G E	360
	BII2HypBA1	367							SE	T	CAA	4 I	S L	A F	F	A R	RM	ILI	EL	A –				· - F	<mark>Y K A</mark> E	390
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	BT_0349	334	Q	<mark>G</mark>					ΡE	T		ΓY	NM	LR		ТΚ	ML	. <mark>Y</mark> () N	S G	DV	DN	SN	N K <mark>F</mark>	<mark>PDP</mark> R	367
GH146 -	BT_0137	344	Т-						GE	T	CC	ΓY	NM	LΚ		S R	ΗL	. F (CW	T -				· - (DSS	368
	XeHypBA1	360	Т-						CE	Н	CA S	SY	NM	LK		T R	ΗL	. Y C	ξW	G -				· - F	<mark>2</mark> Q A E	384
	BII4HypBA1	439	N -						AE	S		AΥ	NM	LK		AR	YL	. F I	- 1	E -				· - (QKPA	463
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	BT 2911	378	к	ASY	YE	RV	LF	NH		s			EK	GN	10	C Y	FT	SN	/ R	PG						413
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GH146 -	XeHypBA1	385	LI	FDY	YE	RT	LL	NH	VN	IAC	QQI	ΗP	RΤ	GM	1 F -	ТΥ	ΜТ	PI	L	AG					- EAR	420
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	BT_2097	447		- <u>к</u> н	I N <mark>R</mark>	W E	WH	GC			<mark>F</mark>	P C	СР	ΡM	1 F	L K	I T	<mark>S</mark> /	۱L	PG	FΙ	Y A	S) <mark>К</mark> -		479
GH127 -	BT_1003	445	- V	N <mark>P</mark> K	E R	ΤE	YI	S C			I	FC	СР	ΡN	1 T	L R	ΤL	. <mark>C</mark> C	Q A	QN	Y A	ΥT	LS	5 <mark>P</mark> -		478
01127 -	BT_3674	391	- (<mark>G</mark> K Y	S R	K P	WF	GC			/	٩C	СР	SΝ	1 V I	<mark>S</mark> R	FΙ	<mark>Р</mark> 3	5 L	ΡG	YV	Y A	V K	(N -		424
	BII1HypBA1	405	- \	V L S	HR	VD	W F	GC			/	A C	СР	AN	11	A R	LI	A S	5 V	DR	ΥI	ΥT	EF	RD-		438
	BII2HypBA1	435	Н١	√ K <mark>P</mark>	VR	QK	W F	GC			/	A C	СР	PN	11	A R		Ε 5	5 V	QE	YA	ΥT	V A	λ E -		469
	BT_2911	414	-	ΙΥΑ	S R	D S	S F				V	vc	CG	нт	G	LE	S P	Ał	(L	SK	FΙ	Y S	HS	5 K F	RIID	449
	BT_0349	404	- \	VY S	QP	ET	SM				V	vC	CV	GS	G	LE	NH	T I	Y	GE	FI	YA	HC	2Q -		435
GH146 -	BT_0137	405	-	LYS	TK	EN	ISF				V	VC	Ľ٧	GS	G	FE	NH	A	Y	GE	AI	ΥY	HN	۱D-		436
-	XeHypBA1	421	- (W S	SP	FD					V	V C		GS	G	ME	AH	AC	ĮΕ	GD	SI	YW	QĽ	G-		452
	BII4HypBA1	500	-)	VNP	AT	QK	EY	GD	GN				LG	GT	A	LE	SH		Y	QD	51	YF		> [-		537
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Figure S1. Amino acid sequence alignment of Bll3HypBA1 and GH127/146 β -Larabinofuranosidases. 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-N Δ 35C Δ 761; lane 3, Bll3HypBA1-N Δ 379C Δ 761; lane 4, N Δ 379C Δ 933 (lane 4). Arrows indicate target proteins at expected molecular size.

Figure S3. Optimal pH and temperature of Bll3HypBA1-N Δ 35C Δ 761. (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-N Δ 35C Δ 761was incubated with L-Ara*f*- β 1,3-L-Ara*f*- α 1,3-Gal- β 1,6-Gal in the presence of 5% methanol, ethanol, or 1-propanol.

Figure S5. Electron density maps.

(A) Zn and coordinating residues in N Δ 379C Δ 761 with a polder map (3 σ). (B) Tris molecule bound to the active site of N Δ 379C Δ 761 with a polder map (4 σ). (C) Anomalous difference maps of the Zn atom. Data collected at 1.280 Å (5 σ , blue) and 1.3000 Å (3.5 σ , green) are shown. (D) Zn and coordinating residues in N Δ 379C Δ 933 with a polder map (3 σ).

Name	Sequence of oligonucleotide primers
$N\Delta$ _Reverse_Primer	5'- CATGGTATATCTCCTTCTTAAAGTT -3'
N∆379rev	5'- GAGAACGTCACCGTAGCCGACGAATA -3'
$C\Delta$ _Forward_Primer	5'-CTCGAGCACCACCACCACCACTG-3'
C∆933rev	5'- GACCTCGGCCATATCCCAGTAGATC -3'
W429A mutation _for	5'- GGCGGT <u>GCG</u> GAGAACGGCCCGGACGAG-3'
W429A mutation _rev	5'- GTTCTC <u>CGC</u> ACCGCCGTAGTTCTTCGC-3'
Y526A mutation _for	5'- CGCTTC <u>GCG</u> AACCTGCACAAGGTTGAG-3'
Y526A mutation _rev	5'- CAGGTT <u>CGC</u> GAACGGCACGATCAGGCC-3'
E578A mutation _for	5'- CGCACT <u>GCG</u> TACGGCGGCATGAATGAC-3'
E578A mutation _rev	5'- GCCGTA <u>CGC</u> AGTGCGCAGCATGTCGGT-3'
H628A mutation _for	5'- GGCTTG <u>GCG</u> GCCAACACCACGATTCCG-3'
H628A mutation _rev	5'- GTTGGC <u>CGC</u> CAAGCCGTTGAGCGGATC-3'
N630A mutation _for	5'- CACGCC <u>GCG</u> ACCACGATTCCGAAGCTC-3'
N630A mutation _rev	5'- CGTGGT <u>CGC</u> GGCGTGCAAGCCGTTGAG-3'
T631A mutation _for	5'- GCCAAC <u>GCG</u> ACGATTCCGAAGCTCACC-3'
T631A mutation _rev	5'- AATCGT <u>CGC</u> GTTGGCGTGCAAGCCGTT-3'
E694Q mutation _for	5'- CAGTCC <u>CAG</u> CACTTCCACGTGGCCGGT -3'
E694Q mutation _rev	5'- GAAGTG <u>CTG</u> GGACTGCGAGTTGCCGCC -3'
E694A mutation _for	5'- CAGTCC <u>GCG</u> CACTTCCACGTGGCCGGT -3'
E694A mutation _rev	5'- GAAGTG <u>CGC</u> GGACTGCGAGTTGCCGCC -3'
E723Q mutation _for	5'- ACCGTG <u>CAG</u> ACCTGCAACGAGTACAAC -3'
E723Q mutation _rev	5'- GCAGGT <u>CTG</u> CACGGTGGAGAAGTTGCG -3'
E723A mutation _for	5'- ACCGTG <u>GCG</u> ACCTGCAACGAGTACAAC -3'
E723A mutation _rev	5'- GCAGGT <u>CGC</u> CACGGTGGAGAAGTTGCG -3'
C725S mutation _for	5'- GAGACC <u>AGC</u> AACGAGTACAACATGCTC -3'
C725S mutation _rev	5'- CTCGTT <u>GCT</u> GGTCTCCACGGTGGAGAA -3'
C725A mutation _for	5'- GAGACC <u>GCG</u> AACGAGTACAACATGCTC -3'
C725A mutation _rev	5'- CTCGTT <u>CGC</u> GGTCTCCACGGTGGAGAA -3'
C804S mutation _for	5'- TATTGG <u>AGC</u> TGCCAGGGTACCGGTATC -3'
C804S mutation _rev	5'- CTGGCA <u>GCT</u> CCAATACTCGCCAATCGC -3'

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

C805S mutation _for	5'- TGGTGC <u>AGC</u> CAGGGTACCGGTATCGAG -3'
C805S mutation _rev	5'- ACCCTG <u>GCT</u> GCACCAATACTCGCCAAT -3'
C805A mutation _for	5'- TGGTGC <u>GCG</u> CAGGGTACCGGTATCGAG -3'
C805A mutation _rev	5'- ACCCTG <u>CGC</u> GCACCAATACTCGCCAAT -3'

The positions of the mutated sequences are underlined.

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Substrates	NΔ35CΔ761	N∆379C∆761	Foldb				
Substrates	(Units/µmol) ^a	(Units/µmol) ^a	1010				
Araf-β1,3-ArafGal 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				

1 **Table S2.** Comparison of the specific activities of Bll3HypBA1-N Δ 35C Δ 761 and

2 Bll3HypBA1-N Δ 379C Δ 761 towards oligosaccharide and polysaccharide.

³ ^aSpecific activity was calculated with the calculated molecular mass of 129,964 Da for

⁴ Bll3HypBA1-N Δ 35C Δ 761 and 94,145 Da for Bll3HypBA1-N Δ 379C Δ 761.

⁵ ^bRatio of the specific activities of Bll3HypBA1-N Δ 35C Δ 761 to Bll3HypBA1-

⁶ ΝΔ379CΔ761.

5 5	0 1		9 1		
Data set	ΝΔ379CΔ761	ΝΔ379CΔ761	ΝΔ379CΔ761	ΝΔ379CΔ761	ΝΔ379CΔ933
	SeMet	+ Tris	Zn peak	Zn low remote	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	$P2_1$	$P2_{1}2_{1}2_{1}$	$P2_1$	$P2_{1}$	$P2_{1}$
Unit cell (Å, °)	<i>a</i> = 59.612, <i>b</i> =	<i>a</i> = 59.592, <i>b</i> = 111.571,	<i>a</i> = 59.582, <i>b</i> =	<i>a</i> = 59.588, <i>b</i> =	<i>a</i> = 75.231, <i>b</i> =
	112.325, $c = 102.747, \beta$	<i>c</i> = 153.010	111.698, <i>c</i> =	111.708, <i>c</i> =	83.510, c = 102.034,
	= 96.306		113.771, $\beta =$	113.778, $\beta =$	$\beta = 90.659$
			104.776	104.781	
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)
$R_{ m pim}$	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)	_

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

completeness	(%)
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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
$R_{\rm work}/R_{\rm free}$		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.

Mutanta	Specific activity	Relative activity ^a
Wittants	(Units/mg)	(%)
Wild type	3.33	100
E694Q	3.63×10 ⁻⁴	0.01
E694A	1.43×10 ⁻⁴	< 0.01
E723Q	5.49×10 ⁻³	0.17
E723A	6.10×10 ⁻⁵	< 0.01
C725S	3.35×10 ⁻¹	10
C804S	2.12×10 ⁻⁴	< 0.01
C805S	1.71×10 ⁻⁴	< 0.01
E694Q/C804S	ND	ND

 Table S4. Specific activities of Bll3HypBA1 mutants.

^aRelative activity was expressed as the percentage of the activity against the wild type Bll3HypBA1-N Δ 379C Δ 933 enzyme.