PNGase H + variant from *Rudaea cellulosilytica* with improved deglycosylation efficiency for rapid analysis of eukaryotic N-glycans and hydrogen deuterium exchange mass spectrometry analysis of glycoproteins

Rui-Rui Guo¹ | Tian-Chan Zhang¹ | Thomas Ole Tandrup Lambert² | Ting Wang¹ | Josef Voglmeir¹ | Kasper D. Rand² | Li Liu¹

¹Glycomics and Glycan Bioengineering Research Center (GGBRC), College of Food Science and Technology, Nanjing Agricultural University, Nanjing, China
²Protein Analysis Group, Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark

Correspondence
J. Voglmeir and L. Liu, Glycomics and Glycan Bioengineering Research Center (GGBRC), College of Food Science and Technology, Nanjing Agricultural University, Nanjing, China. Email: josef.voglmeir@njau.edu.cn and lichen.liu@njau.edu.cn
K. D. Rand, Protein Analysis Group, Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark. Email: kasper.rand@sund.ku.dk

Funding Information
100 Foreign Talents Plan, Grant/Award Number: JSB2014012; H2020 European Research Council, Grant/Award Number: 101003052; National Natural Science Foundation of China, Grant/Award Numbers: 31471703, 31671854, 31871754, 31871793

The analysis of glycoproteins and the comparison of protein N-glycosylation from different eukaryotic origins require unbiased and robust analytical workflows. The structural and functional analysis of vertebrate protein N-glycosylation currently depends extensively on bacterial peptide-N4-(N-acetyl-β-glucosaminyI) asparagine amidases (PNGases), which are indispensable enzymatic tools in releasing asparagine-linked oligosaccharides (N-glycans) from glycoproteins. So far, only limited PNGase candidates are available for N-glycans analysis, and particularly the analysis of plant and invertebrate N-glycans is hampered by the lack of suitable PNGases. Furthermore, liquid chromatography–mass spectrometry (LC–MS) workflows, such as hydrogen deuterium exchange mass spectrometry (HDX-MS), require a highly efficient enzymatic release of N-glycans at low pH values to facilitate the comprehensive structural analysis of glycoproteins. Herein, we describe a previously unstudied superacidic bacterial N-glycanase (PNGase H⁺) originating from the soil bacterium *Rudaea cellulosilytica* (Rc), which has significantly improved enzymatic properties compared to previously described PNGase H⁺ variants. Active and soluble recombinant PNGase Rc was expressed at a higher protein level (3.8-fold) and with higher specific activity (56% increase) compared to the currently used PNGase H⁺ variant from *Dyella japonicum* (Dj). Recombinant PNGase Rc was able to deglycosylate the glycoproteins horseradish peroxidase and bovine lactoferrin significantly faster than PNGase Dj (10 min vs. 6 h). The versatility of PNGase Rc was demonstrated by releasing N-glycans from a diverse array of samples such as peach fruit, king trumpet mushroom, mouse serum, and the soil nematode *Caenorhabditis elegans*. The presence of only two disulfide bonds shown in the AlphaFold protein model (so far all other superacidic PNGases possess more disulfide bonds) could be
1 | INTRODUCTION

N-Glycosylation is a post-translational protein modification that influences a myriad of cellular functions. The comparative analysis of protein N-glycosylation from various sources has become increasingly important and has been reported to be a critical factor in the assessment of proteins in biotechnology, food safety, and structure/function relationship studies. Generally, the analysis of N-glycans requires the enzymatic release of the carbohydrate portion from glycoproteins using PNGases (Figure S1 [supporting information]). The release of vertebrate N-glycans can be achieved by using recombinant PNGase F, which originates from the bacterium Elizabethkingia meningoseptica, and is currently widely applied for the analysis of N-glycans from milk-, egg-, and blood serum glycoproteins. PNGase F can release all types of vertebrate N-glycans (high mannosê-, hybrid-, and complex-type N-glycans), and pre-denaturation of the glycoproteins (i.e., by sodium dodecyl sulfate (SDS) or urea) from the source material greatly enhances the efficiency of the N-glycan release. However, PNGase F is not able to release N-glycans which have an α1,3-linked fucose on the innermost core GlcNAc (“core α1,3-fucose”), and therefore this enzyme is insufficient for releasing N-glycans from invertebrate and plant sources bearing this peculiar glycan modification. A further limitation of PNGase F is the absence of activity in acidic environments as some liquid chromatography–mass spectrometry (LC–MS) workflows, such as hydrogen-deuterium exchange mass spectrometry (HDX-MS), require efficient deglycosylation at low pH values to enable comprehensive structural analysis of glycoproteins.

In contrast to PNGase F, a structurally unrelated enzyme isolated from almond seeds (PNGase A) allows the release of N-glycans from a broad variety of plant and invertebrate glycoproteins. The optimum pH for PNGase A is 5.0, and it shows some residual enzymatic activity even at lower pH values. We previously reported the use of PNGase A to study the conformation and interactions of glycoproteins using HDX-MS. Although PNGase A releases a broad variety of N-glycans from vertebrate, invertebrate, and plant sources, efficient N-glycan release can be achieved only from glycopeptides but not from native glycoproteins, which makes a proteolytic pretreatment of the source materials necessary. Furthermore, PNGase A itself is a glycoprotein and can be self-deglycosylated, and thereby the contamination of glycan samples by endogenous PNGase A glycan structures must be anticipated. So far, approximately 10 PNGase A homologues from plants and fungi have been described yet. However, none of these variants could be successfully expressed in recombinant form in prokaryotic expression systems, thereby limiting their use in glyco-analytical applications.

In recent years, we have worked on the discovery, functional improvement, and applications of a novel class of superacidic bacterial PNGases. Although these enzyme variants could satisfy basic requirements for the release of N-glycans, the recombinant expression levels and overall enzymatic activities could be improved to simplify application within glycoanalytics and LC–MS analysis of glycoproteins. The first reported recombinant variant (originating from Terriglobus roseus, in short, PNGase H⁺ or Tr) was sufficiently active to determine the overall substrate scope, and the basic biochemical parameters such as pH and temperature optima. Its use in glycoproteomic applications such as HDX-MS suffered from low expression yields and purity in comparison to the PNGase variant isolated from almond seeds (PNGase A). One advantage of PNGase Tr relative to PNGase A, however, was a high tolerance to strong reducing agents (e.g., TCEP). Such reducing agents are added during sample preparation during HDX-MS analysis of disulfide-bonded proteins. In a collaborative effort, we then reported the evaluation of 12 more putative PNGase H⁺ variants, of which 3 variants showed significantly higher deglycosylation activities compared to PNGase Tr. The most active variant originating from Dyella japonica (in short, PNGase Dj) showed a fourfold increase in deglycosylation activity of horseradish peroxidase (HRP) when compared to PNGase Tr, and outperformed PNGase A in deglycosylating Trastuzumab glycopeptides. Furthermore, the higher yields and homogeneity after protein purification allowed the immobilization of PNGase Dj on microfluidic chips for studying the glycosylated semi-domain of the tyrosine-protein kinase MET and or the use as an in-situ deglycosylation agent for matrix-assisted laser desorption-mass spectrometry (MALDI)-imaging of N-glycans in soybean root nodules.

Herein, we describe and characterize a previously unstudied recombinant acidic PNGase variant from Rudaea cellulosilytica with improved enzymatic properties and expression yields compared to previously described superacidic PNGases. Our results indicate that PNGase Rc should be a highly suitable enzyme for use in LC/LC–MS workflows for the analysis of released N-linked glycans from a broad array of complex glycoprotein samples as well as for the HDX-MS technique that requires highly efficient deglycosylation at low pH to allow comprehensive analysis of glycoproteins.

2 | METHODS

2.1 | Materials

HRP was obtained from Duly Biotech Company (Nanjing, China). Bovine lactoferrin was purchased from Wako Pure Chemical Industries (Nanjing, China). Peaches (Prunus persica) L. Batsch cvs Xia Hui 6 were obtained from the experimental garden facility of the Jiangsu Academy of Agricultural Sciences (Nanjing, China). King trumpet mushrooms (Pleurotus eryngii) were bought at a local supermarket. C57BL/6 mouse plasma was purchased from Shanghai Fantai Biotechnology Company (Shanghai, China). Caenorhabditis elegans var Bristol strain N2 was correlated by intact mass- and peptide mapping analysis and provides a possible explanation for the improved recombinant expression yield of PNGase Rc.
kindly provided by Dr. Di Chen from the Model Animal Research Center of Nanjing University. Ni-NTA Sefinose Resin was purchased from BBI Life Sciences (Shanghai, China). All other standard chemicals and buffer reagents were of the highest grade available.

2.2 | Plasmid construction, expression, and purification

The candidate gene Rc (from R. cellulolytica DSM 22992, GenBank ID WP_169337280) with Kpn I and Xho I restriction sites was synthesized and ligated into the pET30a vector by Genscript (Nanjing, China). The constructed expression vector was transformed into Escherichia coli BL21 (DE3) competent cells and plated on LB agar supplemented with kanamycin. A single colony was transferred into a 2 L Erlenmeyer shaking flask containing 400 mL of lysogeny broth (LB) medium and shaken at 37°C until the culture density reached an absorbance of 0.5 at 600 nm. The final concentration of 1mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was used to induce the expression of recombinant Rc at a reduced temperature of 18°C. After an induction period of 20 h, cells were collected by centrifuging at 12 000 g for 20 min. The resulting cell pellet was resuspended in 10 mL of lysis buffer (100mM, NaCl, 50mM Tris, 1% [v/v] Triton X-100, 1mM PMSF, adjusted to pH 8.0) and sonicated for 30 min. The cell lysates were centrifuged for 20 min at 12 000g, and then the supernatant was applied onto a Ni-NTA column (10 mL of bed volume). About 200 mL of washing buffer (consisting of 50mM NaCl, and 50mM Tris, adjusted to pH 8.0 with HCl) was used to wash off unspecifically retained proteins. A simple one-step purification with 20 mL of elution buffer containing 500mM imidazole was directly used to elute target proteins. Samples were further analyzed using SDS-PAGE, and the fractions including candidate protein were stored at 4°C for further experiments.

2.3 | Site-directed mutagenesis of PNGase Rc

PNGase Rc site-directed mutagenesis was generated according to the QuickChange XL site-directed mutagenesis protocol (Stratagene) using the former primer 5'-ACCGGTACTCGTACGACGGCGTACCGCTGATCT-G-3'and the reverse primer 5'-CACATACGAGCGGTACGGCGTACTGACGAGTACCGGT-3'. The mutated plasmid was verified using DNA sequencing and transformed into E. coli BL21 (DE3) competent cells for recombinant expression. The expression and purification was performed under the conditions mentioned earlier (Section 2.2). The activity test of PNGase Rc mutant was carried out using gel-based deglycosylation assay.39

2.4 | Substrate specificity of PNGase Rc

HRP and bovine lactoferrin were selected to evaluate substrate specificity of recombinant PNGase Rc. About 10μL of each glycoprotein (20 μg/μL) was incubated with 190 μL of PNGase Rc reaction mixture (consisting of 10 μg of PNGase Rc and 300mM of citrate/sodium phosphate buffer [pH 2.0] in water). After the reaction mixtures were incubated for 1 h at 37°C, the samples were centrifuged for 5 min at 12 000g, and the supernatant was purified with solid-phase extraction (Supelclean ENVI-Carb SPE Tube, 3 mL, 250 mg, No 57088). The purified N-glycans were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in a positive ion mode using 2, 5-dihydroxybenzoic acid (10 mg/mL in acetonitrile) as a matrix. The acquired data were processed using the Bruker Flex Analysis software (version 3.3).

2.5 | Peptide mapping

Peptic digestion of reduced PNGase was performed by first diluting 5 μL of 200 pmol PNGase Rc 1:1 with 300mM phosphate buffer (pH 2.3), 6M guanidinium hydrochloride (GdnHCl), and 0.5M TCEP.

FIGURE 1 Comparison of cysteine abundance in PNGase variants. Cysteines are shown in yellow (participating in disulfide bond formation) and green (not participating in disulfide bond formation). The protein models were generated and visualized using the AlphaFold toolset of the UCSF ChimeraX software suite (www.rbvi.ucsf.edu/chimerax) with the respective PNGase amino acid sequences obtained from GenBank (accession codes for Dj: WP_019464163.1, Tr: WP_014787206.1, Rc: WP_169337280.1, and A [from almonds]: XP_034202935) [Color figure can be viewed at wileyonlinelibrary.com]
The solution was incubated at 37°C for 30 min. About 90 μL of solvent A (0.23% formic acid) was added prior to injection. For non-reduced samples, the 0.5M TCEP was not added. Digestion was performed on a home-packed pepsin column (internal volume of 60 μL) coupled to the LC-MS system upstream of the C18 trap column (see below). For tryptic digestion of reduced Rc, 5 μL of 200 pmol of PNGase Rc was diluted in 10 μL of 6M GdnHCl, 50mM NH₄HCO₃ (pH 8.00), and the resulting mixture was incubated at 37°C for 30 min. About 90 μL of solvent A (0.23% formic acid) was added prior to injection. For non-reduced samples, the 0.5M TCEP was not added. Digestion was performed on a home-packed pepsin column (internal volume of 60 μL) coupled to the LC-MS system upstream of the C18 trap column (see below). For tryptic digestion of reduced Rc, 5 μL of 200 pmol of PNGase Rc was diluted in 10 μL of 6M GdnHCl, 50mM NH₄HCO₃ (pH 8.00), and the resulting mixture was incubated at

(A) Horseradish Peroxidase Digests

(B) UPLC Analysis of Horseradish Peroxidase N-Glycans

(C) MALDI-ToF-MS of Horseradish Peroxidase N-Glycans

(D) Bovine Lactoferrin Digests

(E) UPLC Analysis of Bovine Lactoferrin N-Glycans

(F) MALDI-ToF-MS of Bovine Lactoferrin N-Glycans

FIGURE 2 PNGase Rc catalyzed N-glycan release from horseradish peroxidase (HRP) and bovine lactoferrin. Time course analysis of the deglycosylation reaction of HRP using (A), an SDS-PAGE shift assay and (B) and (C), ultra-performance liquid chromatography (UPLC)- and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS)-based analysis of the released N-glycans. Panels (D), (E), and (F) used bovine lactoferrin as glycoprotein substrate. The red arrow marks the protein band of PNGase Rc in the assay mixtures. The substitution of the aspartic acid residue at position 106 with alanine (RcD106A) rendered this mutant variant inactive. N-Glycans are depicted in the symbol nomenclature for glycans (SNFG) [Color figure can be viewed at wileyonlinelibrary.com]
60°C for 30 min. The sample was reduced by adding 5 μL of 45mM dithiothreitol (DTT) and incubated at 60°C for 3 h. Reduced cysteines were alkylated by adding 5 μL of 100mM iodoacetamide (IAM) and incubated at room temperature for 30 min. About 7.7 μL of 650mM CaCl2 was added to assist tryptic stability. The sample was diluted to 100 μL with 50mM NH₄HCO₃ (pH 8.00), and 5μM of trypsin was added in a w/w relationship of 1:20 and incubated overnight at 37°C. For non-reduced samples, DTT was replaced with MilliQ water.

LC-MS was performed by loading samples onto a UPLC-MS system where desalting was performed via a C18 vanguard trap column with a 200 μL/min flow rate of solvent for 3 min. After desalting, peptides were separated on a C18 analytical column and were eluted off column through a gradient of solvent B (5% to 95% over 9 min). MS and MS/MS analysis was performed on a hybrid ESI-Q-TOF mass spectrometer (Synapt G2-Si). Data analysis was performed using Maxent1 function of the Masslynx software.

3 | RESULTS AND DISCUSSION

Prior to this work, the PNGase H⁺ variant reported to have the highest recombinant expression levels in E. coli was PNGase Dj. By comparing AlphaFold-derived protein structure models, we noticed that PNGase Dj had fewer cysteine residues compared with PNGase Tr (six versus eight cysteine residues), of which six were predicted to form three intramolecular disulfide bonds (Figure 1). Given that cytosolic expression in E. coli is not optimal for the expression of proteins containing disulfide bonds, we speculated that a lower number of cysteine residues present in the PNGase H⁺ protein sequence may be beneficial for the soluble expression of functional PNGases.

A BLAST search of putative superacidic PNGases from bacterial genome databases identified a variant from R. cellulosilytica, which possessed only four cysteine residues in its protein sequence (Table S1 [supporting information]), and therefore it was chosen as a candidate for initial expression experiments and activity studies. Deglycosylation experiments showed that PNGase Rc was able to
fully deglycosylate the glycoprotein substrates HRP and bovine lactoferrin within 10 min of incubation time (Figure 2). Notably, a variant of Rc where the aspartic acid residue at position 106 was substituted with alanine (RcD106A) was inactive (Figure 2), indicating the importance of this residue for the catalytic activity of Rc.

Deglycosylation of PNGase Rc was significantly faster than the currently used PNGase Tr and Dj variants, which deglycosylated lactoferrin in an identical assay within 6 h and “overnight,” respectively. A comparison of the overall amount of purified recombinant proteins showed that PNGase Rc yielded 3.8 times more protein than PNGase Dj (5.3 ± 0.5 mg of purified PNGase Rc and 1.4 ± 0.2 mg of PNGase Dj from 400 mL of expression culture, respectively, Figure S2 [supporting information]). In addition, PNGase Rc also showed a ~56% increased specific activity compared to PNGase Dj using a colorimetric HRP deglycosylation assay (122 ± 6 U/mg and 78.1 ± 3.7 U/mg, respectively, Figure S3 [supporting information]).

We next characterized the primary structure of PNGase Rc using LC–MS (Figure 3). Mass analysis of the native intact (unreduced) PNGase Rc yielded a mass of 64199.0 Da. This experimental mass is in good agreement (within a mass accuracy of 17 ppm) with an expected theoretical mass of 64197.9 Da based on the cloned sequence and with the assumption that all four C residues of Rc participate in disulfide bonds. In support of this finding, the AlphaFold model of PNGase Rc indeed indicated two disulfide linkages in Rc, between C58-C84 and C554-574 (Figure 1). To confirm the presence of the two disulfide bonds in Rc, we also performed mass analysis of intact Rc by treating it with the reducing agent TCEP, which yielded a mass of 64202.9 Da corresponding to the expected mass shift due to reduction of four cysteine residues (Figure S5 [supporting information]).
The primary structure of Rc was further verified by proteolysis of unreduced and reduced Rc using either pepsin or trypsin and using LC–MS/MS to identify and map the resulting peptides to the expected sequence of Rc (Figures S6–S8 [supporting information]). The peptide mapping analysis using pepsin of reduced and unreduced Rc yielded peptides confirming 81% and 75% of the sequence, respectively, including the N- and C-termini. The corresponding peptide maps using trypsin provided sequence coverages of 43% and 47%, respectively, including covering the N- and C-termini. Thus, the data from intact mass analysis and peptide mapping collectively confirmed the sequence of Rc. In addition, peptide mapping of unreduced Rc using trypsin identified a disulfide-linked peptide consisting of tryptic peptides 526–556 (α) and 564–589 (β) (Figure 4). The summed overall sequence coverage from all the approaches was 90.4%.

Importantly, this peptide was absent in the corresponding reduced sample. Our experimental data thus directly confirmed a C554–C574 disulfide bond in Rc—and by inference thus also a disulfide bond between the two remaining C residues (C58-C84) (Figure S9 [supporting information]).

To evaluate the deglycosylation activity of PNGase Rc, a variety of sample materials were subjected to an enzymatic N-glycan release. UPLC analysis revealed that the N-glycan profiles from king trumpet mushroom and mouse serum samples treated with PNGase Rc were very similar to the N-glycan profiles obtained using PNGase F (Figures 5A and 5B), and showed the expected signature pattern for the N-glycosylation of fungi (mostly high-mannose type46,47) and mammals (mostly complex type N-glycans48). As anticipated, the N-glycan profiles obtained from the peach fruit and C. elegans samples showed a significantly higher N-glycan variety for PNGase Rc–treated samples (Figures 5C and 5D), which is caused by the inability of PNGase F to release N-glycans bearing core α1,3-fucose moieties, which are present in both plant N-glycans and invertebrate N-glycans.29,49

To further evaluate the effect of the acidic reaction conditions on acid-labile carbohydrate moieties such as terminally linked sialic acid residues, sialylated glycoproteins such as human immunoglobulin G (IgG) or bovine fetuin were also incubated in a short time deglycosylation experiment (1 h enzymatic N-glycan release) using PNGase Rc. UPLC and MALDI-TOF analysis showed that the release efficiency of PNGase Rc is comparable to PNGase F, and that no loss of sialic acid residues occurred during the enzymatic release (Figure S10 [supporting information]).
4 | CONCLUSIONS

Here we express and characterize a previously unstudied superacidic bacterial N-glycanase (of the PNGase H\(^+\) family) that originates from the soil bacterium \textit{R. cellulolytica} (Rc). We show that the enzyme has significantly improved enzymatic properties for use in workflows for glycan and glycoprotein analysis, compared to previously described PNGase H\(^+\) variants. PNGase Rc can be expressed recombinantly and purified at significantly higher yield and concentrations, and also possesses a higher specific activity levels than the previously studied superacidic PNGases. We anticipate that the PNGase Rc will be of particular benefit to research within the fields of glycomics, glycoproteomics, and HDX-MS. We note that PNGase Rc could, for instance, be readily used instead of PNGase A or PNGase Dj in our previously reported HDX-MS workflows for analysis of glycoproteins. \(^{30,40}\)

ACKNOWLEDGMENTS

This work was supported in part by the European Research Councils (ERC Consolidator Grant no. 101003052, to K.D.R.), National Natural Science Foundation of China (grant numbers 31471703, 31671854, 31871793, and 31871754 to J.V. and L.L.), and the 100 Foreign Talents Plan (grant number JSB2014012 to J.V.). During review of this manuscript, a manuscript by Gramlich et al was published describing use of PNGase Rc for similar LC-MS workflows as described here.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/rcm.9376.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Josef Voglmeir \(\text{https://orcid.org/0000-0002-4096-4926}\)
Kasper D. Rand \(\text{https://orcid.org/0000-0002-6337-5489}\)
Li Liu \(\text{https://orcid.org/0000-0002-2178-9237}\)

REFERENCES

1. Mastrangeli R, Audino MC, Palinsky W, Broly H, Bierau H. The formidable challenge of controlling high mannosetype N-Glycans in therapeutic mAbs. Trends Biotechnol. 2020;38(10):1154-1168. doi:10.1016/j.tibtech.2020.05.009
2. Sha S, Agarabi C, Bronson K, Lee DY, Yoon S. N-glycosylation design and control of therapeutic monoclonal antibodies. Trends Biotechnol. 2016;34(10):835-846. doi:10.1016/j.tibtech.2016.02.013
3. Wang T, Liu L, Voglmeir J. mAbs N-glycosylation: Implications for biotechnology and analytics. Carbohydr. Res. 2022;514:108541. doi:10.1016/j.carres.2022.108541
4. Wang T, Hu XC, Cai ZP, Voglmeir J, Liu L. Qualitative and quantitative analysis of carbohydrate modification on glycoproteins from seeds of Ginkgo biloba. J Agric Food Chem. 2017;65(35):7669-7679. doi:10.1021/acs.jafc.7b01690
5. Shi ZH, Yin BR, Li YQ, et al. N-glycan profile as a tool in qualitative and quantitative analysis of meat adulteration. J Agric Food Chem. 2019;67(37):10543-10551. doi:10.1021/acs.jafc.9b03756
6. Meli VS, Ghosh S, Prabha TN, Chakraborty N, Chakraborty S, Datta A. Enhancement of fruit shelf life by suppressing N-glycan processing enzymes. Proc Natl Acad Sci U S A. 2010;107(6):2413-2418. doi:10.1073/pnas.0909329107
7. Wang WL, Wang W, Du YM, et al. Comparison of anti-pathogenic activities of the human and bovine milk N-glycane: Fucosylation is a key factor. Food Chem. 2017;235:167-174. doi:10.1016/j.foodchem.2017.05.026
8. Wang WL, Du YM, Wang W, et al. Comparison of the bifidogenic activity of human and bovine milk N-glycome. J Funct Foods. 2017;33:40-51. doi:10.1016/j.jff.2017.03.017
9. Zheng F, Du YM, Lin XS, et al. N-glycosylation plays an essential and species-specific role in anti-infection function of Milk proteins using listeria monocytogenes as model pathogen. J Agric Food Chem. 2019;67(38):10774-10781. doi:10.1021/acs.jafc.9b03154
10. Wang T, Jia X-R, Liu L, Voglmeir J. Changes in protein N-glycosylation during the fruit development and ripening in melting-type peach. Food Mat Res. 2021;1(1):2-8. doi:10.48130/FMR-2021-0002
11. Plummer TH Jr, Elder JH, Alexander S, Phelan AW, Tarentino AL. Demonstration of peptide: N-glycosidase F activity in endo-beta-N-acetylglucosaminidase F preparations. J Biol Chem. 1984;259(17):10700-10704. doi:10.1016/S0021-9258(18)90568-5
12. Valk-Weeber RL, Eshuis-de Ruiter T, Dijkhuisen L, van Leeuwen SS. Dynamic temporal variations in bovine Lactoferrin glycan structures. J Agric Food Chem. 2020;68(2):549-560. doi:10.1021/acs.jafc.9b06762
13. Meng Y, Qiu N, Geng F, Huo Y, Sun H, Keast R. Identification of the duck egg white N-Glycoproteome and insight into the course of biological evolution. J Agric Food Chem. 2019;67(35):9950-9957. doi:10.1021/acs.jafc.9b03059
14. Zhang Y-Y, Ghirardello M, Wang T, et al. Imidazolium labelling permits the sensitive mass-spectrometric detection of N-glycosides directly from serum. Chem Commun (Camb). 2021;57(57):7003-7006. doi:10.1039/D1CC02100A
15. Vilaj M, Lauc G, Trbojevičič I. Evaluation of different PNGase F enzymes in immunoglobulin G and total plasma N-glycans analysis. Glycobiology. 2021;31(1):2-7. doi:10.1093/glycob/cwa047
16. Gnanesh Kumar BS, Mohan Reddy P, Kottekad S. Comparative site-specific N-glycosylation analysis of Lactoperoxidase from Buffalo and goat Milk using RP-UHPLC-MS/MS reveals a distinct glycan pattern. J Agric Food Chem. 2018;66:11492-11499. doi:10.1021/acs.jafc.8b02243
17. Chen C, Li T, Chen G, et al. Commensal relationship of three bifidobacterial species leads to increase of Bifidobacterium in vitro fermentation of Sialylated immunoglobulin G by human gut microbiota. J Agric Food Chem. 2020;68(34):9110-9119. doi:10.1021/acs.jafc.0c03628
18. Mu C, Cai Z, Bian G, et al. New insights into porcine Milk N-Glycome and the potential relation with offspring gut microbiome. J Proteome Res. 2019;18(3):1114-1124. doi:10.1021/acs.jproteome.8b00779
19. Blanchard V, Frank M, Leeflang BR, Boelens R, Kamerling JP. The structural basis of the difference in sensitivity for PNGase F in the de-N-glycosylation of the native bovine pancreatic ribonuclease B and BS. Biochemistry. 2008;47(11):3435-3446. doi:10.1021/bi7012504
20. Tarentino AL, Gómez CM, Plummer TH Jr. Deglycosylation of asparagine-linked glycosides by peptide: N-glycosidase F. J Agric Food Chem. 1985;33(17):4665-4671. doi:10.1021/bi00338a028
21. Yan S, Vanbeselaere J, Jin C, et al. Core richness of N-Glycans of \textit{Caenorhabditis elegans}: A case study on chemical and enzymatic release. Anal Chem. 2018;90(1):928-935. doi:10.1021/acs.analchem.7b03898
22. Tretter V, Alltmann F, Márz L. Peptide-N4-(N-acetyl-beta-glucosaminyI)asparagine amidase F cannot release glycans with fucose attached alpha1 3 to the asparagine-linked N-
acetylglucosamine residue. *Eur J Biochem*. 1991;199:647-652. doi:10.1111/j.1432-1033.1991.tb16166.x

23. Trabjerg E, Nazeri ZE, Rand KD. Conformational analysis of complex protein states by hydrogen/deuterium exchange mass spectrometry (HDX-MS): Challenges and emerging solutions. *Trends Anal Chem*. 2018;106:125-138. doi:10.1016/j.trac.2018.06.008

24. Takahashi N. Demonstration of a new amidase acting on glycopeptides. *Biochem Biophys Res Commun*. 1977;76(4):1194-1201. doi:10.1016/0006-291X(77)90982-2

25. Takahashi N, Nishibe H. Some characteristics of a new glycopeptidase acting on aspartylglucosaminyl linkages. *J Biochem*. 1978;84(6):1467-1473. doi:10.1093/oxfordjournals.jbchem.a132270

26. Jensen PF, Comamala G, Trelle MB, Madsen JB, Jørgensen TJ, Rand KD. Removal of N-linked Glycosylations at acidic pH by PNGase F facilitates hydrogen/deuterium exchange mass spectrometry analysis of N-linked glycoproteins. *Anal Chem*. 2016;88(24):12479-12488. doi:10.1021/acs.analchem.6b03951

27. Fan JQ, Lee YC. Detailed studies on substrate structure requirements of glycoamidases A and F. *J Biol Chem*. 1997;272(43):27058-27064. doi:10.1074/jbc.272.43.27058

28. Plummer TH Jr, Tarentino AL. Cleavage of complex oligosaccharides from glycopeptides by almond emulsion peptide N-glycosidase. *J Biol Chem*. 1981;256(20):10243-10246. doi:10.1016/0021-9258(81)90509-9

29. Altman F, Paschinger K, Dallik T, Vorauer K. Characterisation of peptide-N\(^\beta\)-N-acetylglucosaminyl asparagine amidase A and its N-glycans. *Eur J Biochem*. 1998;252(1):118-123. doi:10.1046/j.1432-1327.1998.2520118.x

30. Plummer TH Jr, Phelan AW, Tarentino AL. Detection and quantification of peptide-N\(^\beta\)-N-acetylglucosaminyl) asparagine amidases. *Eur J Biochem*. 1987;163(1):167-173. doi:10.1111/j.1432-1033.1997.tb10751.x

31. Hossain MA, Nakano R, Nakamura K, Kimura Y. Molecular identification and characterization of an acidic peptide: N-glycanase from tomato (*Lycopersicum esculentum*) fruits. *J Biol Chem*. 2010;147(2):157-165. doi:10.1093/jbc/mvp157

32. Lherould S, Karamanos Y, Lerouge P, Morvan H. Characterization of the N-acetylglucosaminyl asparagine amidase (PNGase F) from *Alkaligenic species* aspartylglucosaminyl linkages. *Cell Proteomics*. 1993;1(1):94-98. doi:10.1074/mcp.M115.055061

33. Sugiyama K, Ishihara H, Tejima S, Takahashi N. Demonstration of a new glycopeptidase from jack-bean meal, acting on aspartylglycosylamine linkages. *Biochem Biophys Res Commun*. 1983;112(1):155-160. doi:10.1016/0006-291X(83)91810-7

34. Chang T, Kuo MC, Khoo KH, Inoue S, Inoue Y. Developmentally regulated expression of a peptide: N-glycanase during germination of rice seeds (*Oryza sativa*) and its purification and characterization. *J Biol Chem*. 2000;275(12):129-134. doi:10.1074/jbc.275.129

35. F louhi-Paquin N, Hauer CR, Stack RF, Tarentino AL, Plummer TH Jr. Molecular cloning, primary structure, and properties of a new glycoamidase from the fungus aspergillus tubigensis. *J Biol Chem*. 1997;272(36):22960-22965. doi:10.1074/jbc.272.36.22960

36. Comamala G, Krogh CC, Nielsen VS, Kutter JP, Voglmeyer J, Rand KD. Hydrogen/deuterium exchange mass spectrometry with integrated electrochemical reduction and microchip-enabled Deglycosylation for epitope mapping of heavily glycosylated and disulfide-bonded proteins. *Anal Chem*. 2021;93(49):16330-16340. doi:10.1021/acs.analchem.1c04172

37. Du YM, Xia T, Gu XQ, et al. Rapid sample preparation methodology for plant N-glycan analysis using acid-stable PNGase H\(^+\). *J Agric Food Chem*. 2015;63(48):10550-10555. doi:10.1021/acs.jafc.5b03633

38. Du YM, Zheng SL, Liu L, Voglmeyer J, Yeidt G. Analysis of N-glycans from *Raphanus sativus* cultivars using PNGase H. *J vis Exp*. 2018;25:57979.

39. Guo RR, Comamala G, Yang HH, et al. Discovery of highly active recombinant PNGase H\(^+\) variants through the rational exploration of unstudied Acidobacterial genomes. *Front Bioeng Biotechnol*. 2020;8:741. doi:10.3389/fbioe.2020.00741

40. Comamala G, Madsen JB, Voglmeyer J, et al. Deglycosylation by the acidic glycosidase PNGase H\(^+\) enables analysis of N-linked glycoproteins by hydrogen/deuterium exchange mass spectrometry. *J Am Soc Mass Spectrom*. 2020;31(11):2305-2312. doi:10.1012/jams.0c00258

41. Velickovic D, Yen-Chen L, Thibert S, et al. Spatial mapping of plant N-glycosylation cellular heterogeneity inside soybean root nodules provided insights into legume-rhizobia symbiosis. *Front Plant Sci*. 2022;13:869281. doi:10.3389/Fpls.2022.869281

42. Jumper J, Evans R, Pritzl A, et al. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596(7873):583-589. doi:10.1038/s41586-021-03819-2

43. Stewart EJ, Aslund F, Beckwith J. Disulfide bond formation in the Escherichia coli cytoplasm: An in vivo role reversal for the threoredoxins. *EMBO J*. 1998;17(19):5543-5550. doi:10.1093/emboj/17.19.5543

44. Wang T, Cai ZP, Gu XQ, et al. Discovery and characterization of a novel extremely acidic bacterial N-glycanase with combined advantages of PNGase F and α. *Biosci Rep*. 2014;34(6):e00149. doi:10.1042/BSR20140148

45. Wang T, Zheng SL, Liu L, Voglmeyer J. Development of a colorimetric PNGase activity assay. *Carbohydr Res*. 2019;472:58-64. doi:10.1016/j.carres.2018.11.007

46. Berends E, Ohm RA, de Jong JF, et al. Genomic and biochemical analysis of N-glycosylation in the mushroom-forming basidiomycete *Schizopyllum commune*. *Appl Environ Microbiol*. 2009;75(13):4648-4652. doi:10.1128/AEM.00352-09

47. Hykollari A, Eckmair B, Voglmeyer J, et al. More than just N-glycosylation: Improved deglycosylation efficiency for rapid analysis of eukaryotic N-glycans with PNGase H\(^+\). *PLoS One*. 2013;8(9):e71159. doi:10.1371/journal.pone.0071159

48. Paschinger K, Wilson IBH. Comparisons of N-glycans across invertebrate phyla. *Parasitolology*. 2019;146(14):1733-1742. doi:10.1017/S003118201900398

**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Guo R, Zhang T-C, Lambert TOT, et al. PNGase H\(^+\) variant from *Rudaea cellulosilytica* with improved deglycosylation efficiency for rapid analysis of eukaryotic N-glycans and hydrogen deuterium exchange mass spectrometry analysis of glycoproteins. *Rapid Commun Mass Spectrom*. 2022;36(21):e9376. doi:10.1002/rcm.9376