Optimizing reaction conditions for deglycosylation of intact proteins using a novel enzyme PNGase Rc

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Introduction

One of the most complex and diverse PTMs of proteins is glycosylation, which involves the covalent attachment of glycan molecules to asparagine (N-glycosylation) or serine/threonine (O-glycosylation) (1). Protein glycosylation has a profound impact on protein structure, stability, solubility, folding, trafficking, and interaction with other molecules (2, 3).

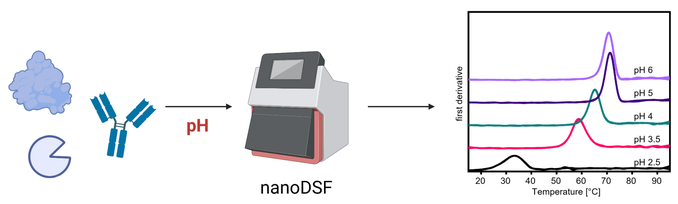
N-glycosylation poses a challenge for the mass spectrometric analysis of proteins due to its heterogeneity. Specifically, this complicates protein identification due to the distribution of signals across multiple glycoprotein species with unknown N-glycan content and structure (4, 5). Commercially available peptide N-glycanases (PNGases) enable protein deglycosylation and are widely used in biopharmaceutical protein characterization.

For efficient deglycosylation, these enzyme kits often use detergent-containing buffers to unfold proteins and expose their N-glycosylation sites (6). Whereas commercially available PNGases work at neutral pH, the novel PNGase Rc produced in-house deglycosylates proteins at acidic pH (7). Here, we investigated whether this condition contributes to protein unfolding and therefore enables deglycosylation under detergent-free, mass spectrometry-friendly conditions.

Multiple techniques including nanoDSF, gel electrophoresis and mass spectrometry were used to optimize the reaction conditions for deglycosylation of intact antibodies and other proteins with complex glycan structures under non-reducing, detergent-free, acidic conditions.

Workflow

Thermal stability assessment Tm(pH)



Enzyme activity assessment EA(t, T, E:S)

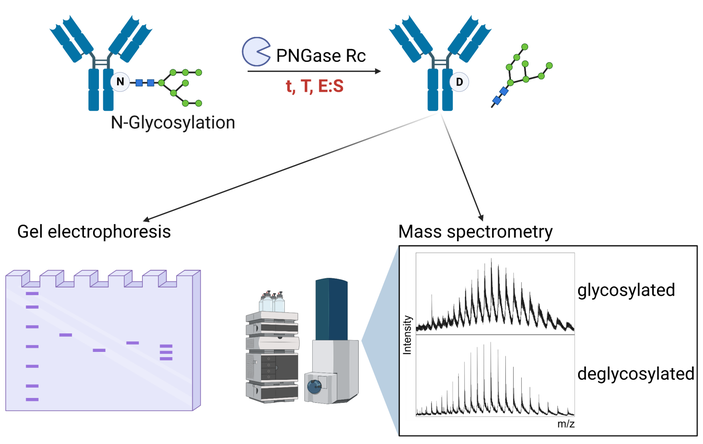


***Figure 1:* Workflow for parameter assessment for optimal PNGase Rc enzyme activity: pH, t, T, E:S).** Created with BioRender.

Thermal stability of PNGase Rc

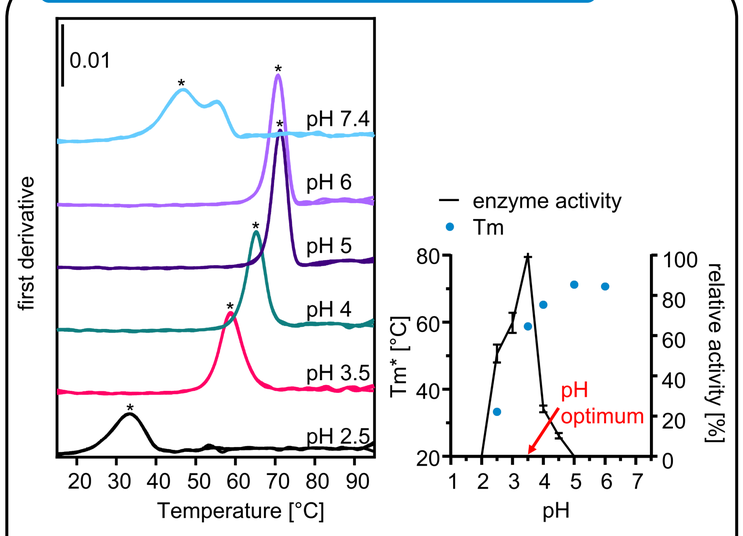
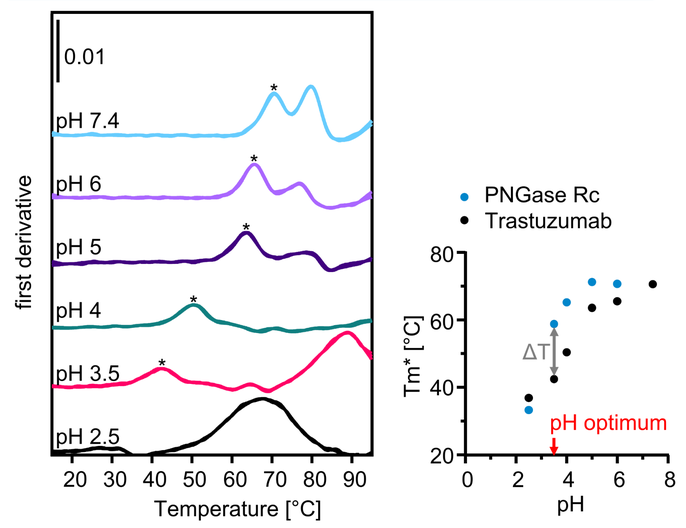
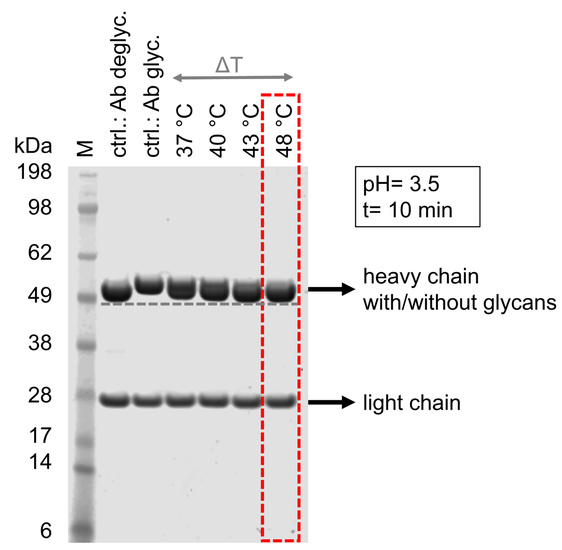


Figure 2:* Thermal unfolding and enzyme activity of PNGase Rc at different pH values.** PNGase Rc (250 µg/mL) was diluted in 100 mM ammonium formate buffer (pH 2.5, 3.5), 100 mM citrate-NaOH buffer (pH 4, 5), 20 mM histidine/ 140 mM NaCl buffer (pH 6) and 1x PBS (pH 7.4). (left***) The first derivative of the ratio Em_{350nm}/Em_{330nm} is plotted against the temperature. (***right***) Melting temperature (Tm) is plotted against the pH and determination of pH optimum is shown (7).

Antibody deglycosylation conditions





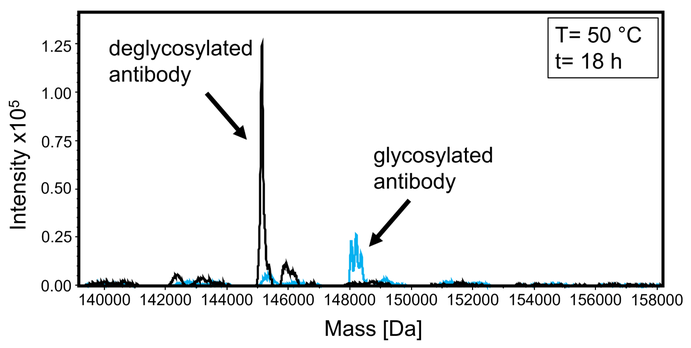
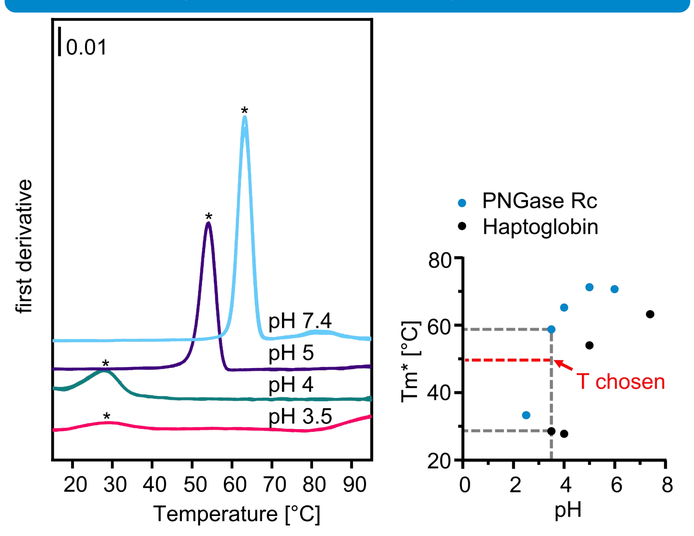
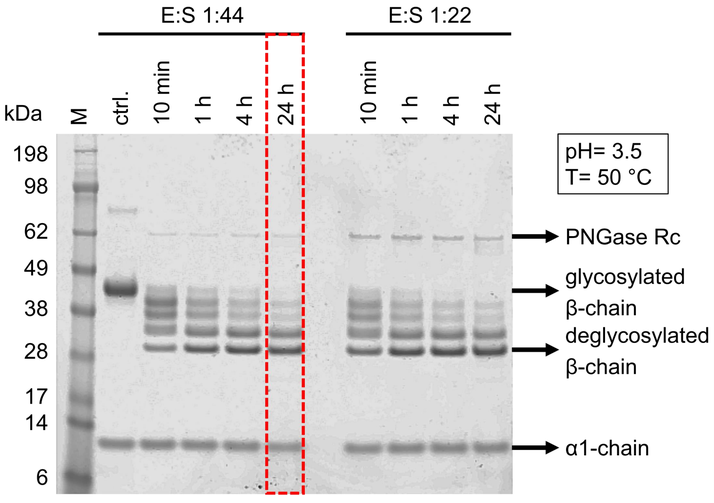


Figure 3:* Thermal unfolding and deglycosylation of trastuzumab.** (**top**) Trastuzumab (500 µg/mL) was diluted in 100 mM ammonium formate buffer (pH 2.5, 3.5), 100 mM citrate-NaOH buffer (pH 4, 5), 20 mM histidine/ 140 mM NaCl buffer (pH 6) and 1x PBS (pH 7.4). (left***) The first derivative of the ratio Em_{350nm}/Em_{330nm} is plotted against the temperature. (***right***) Melting temperature is plotted against the pH. (**middle**) Trastuzumab was incubated with PNGase Rc at pH 3.5 (E:S = 1:48 (M:M), T= 37, 40, 43, 48 °C, t= 10 min). (**bottom**) Overlay of charge-deconvoluted mass spectra showing fully (black) and non-deglycosylated (blue) IgG molecules after incubation with PNGase Rc at 50 °C for 18 h.

Complex glycoprotein deglycosylation





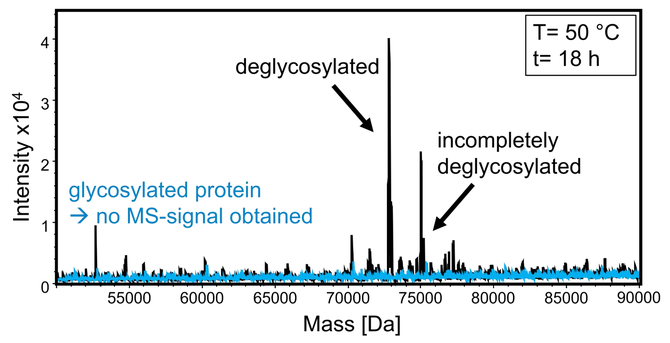


***Figure 4:* Thermal unfolding and deglycosylation of haptoglobin.** (**top**) Haptoglobin (500 µg/mL) was diluted in 100 mM ammonium formate buffer (pH 3.5), 100 mM citrate-NaOH buffer (pH 4, 5) and 1x PBS (pH 7.4). The first derivative of the ratio Em_{350nm}/Em_{330nm} is plotted against the temperature. (**middle**) Haptoglobin was incubated with PNGase Rc at pH 3.5 (E:S = 1:48 and 1:22 (M:M), T= 50 °C) with varying incubation periods (t= 10 min, 1, 4, 24 h). (**bottom**) Overlay of charge-deconvoluted mass spectra showing fully/partially deglycosylated (black) and glycosylated (blue) haptoglobin after incubation with PNGase Rc at 50 °C for 18 h.

Summary and Outlook

- The combination of **acidic pH** and elevated **temperature** induces **protein unfolding** and facilitates deglycosylation of intact proteins due to the simultaneously high thermal stability under acidic conditions of PNGase Rc.
- Determination of **Tm at different pH values** allowed a straightforward optimization of the multiparametric deglycosylation conditions.
- Novel PNGase Rc shows the best enzymatic activity regarding the deglycosylation of intact proteins: **pH=3.5, T=50 °C, E:S 1:44, t=10 min** for Ab and **=18 h** for complex glycoprotein.
- Implementation of protein deglycosylation into routine mass spectrometry analysis with **detergent-free** sample preparation achieved.
- **On-line** deglycosylation is under development.

Acknowledgements

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Baden-Württemberg

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