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Serine scanning—A tool to prove the consequences of *N*-glycosylation of proteins

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Abstract

N-Glycosylation of proteins is a common posttranslational modification in eukaryotes. Often this results in enhanced protein stability through protection by the attached sugar moieties. Due to its 13 potential *N*-glycosylation motifs (N-X-T/S), recombinant hydroxynitrile lyase isoenzyme 5 from almonds (*Pa*HNL5) is secreted by the heterologous host *Pichia pastoris* in a massively glycosylated form, and it shows extraordinary stability at low pH. The importance of *N*-glycosylation in general, and individual glycosylation sites in particular for stability at low pH were investigated. To identify especially important glycosylation sites asparagine from all N-X-S/T-motifs was replaced by serine. Thus, critical sites, which contributed to overall enzyme activity and/or stability, were identified individually. One glycosylation site revealed to be essential for stability at low pH. After enzymatic deglycosylation, leaving only one acetylglucosamine attached to asparagines, *Pa*HNL5 retained most of its stability at low pH. Protonation effects in the active site as well as higher-order aggregational events upon incubation in low pH were excluded. This study provides evidence for the interconnection of *N*-glycosylation and stability at low pH for *Pa*HNL5. Moreover, serine scanning was proven to be applicable for quick identification of critical glycosylation sites.

Keywords: pH stability; *N*-Glycosylation; Serine scanning; *Pichia pastoris*; Hydroxynitrile lyase (HNL); Tunicamycin

1. Introduction

Glycosylation is regarded as one of the most important posttranslational modifications in newly synthesized proteins (Lis and Sharon, 1993; Varki, 1993).

N-Glycosylation is a co-translational event where prefabricated oligosaccharides units are specifically transferred to the growing polypeptide chain as soon as it enters the lumen of the endoplasmatic reticulum (Woods et al., 1994). The side-chain carboxamide group of asparagine serves as acceptor for attached oligosaccharides if present in the particular sequon N-X-T/S-Y, whereby Pro at positions X or Y completely inhibits the decoration with oligosaccharides (Kasturi et al., 1995; Shakin-Eshleman et al., 1996).

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N-Glycosylation of proteins can influence conformational dynamics of nascent polypeptides, thus controlling protein folding (O'Connor and Imperiali, 1996). Moreover, oligosaccharide chains act as a shield around the peptide and thereby increase thermal stability (Kato et al., 2000; Wang et al., 1996), solubility (Kundra and Kornfeld, 1999), resistance to proteolytic digestion (Stochaj et al., 1992; van Berkel et al., 1995), overall rigidity (Rudd et al., 1994) and stability at acidic or basic pH-values (Khan et al., 2003) of the protein. Furthermore, the specific activity of enzymes can be influenced by attached sugar moieties, as well (Khan et al., 2003). In general terms, attached carbohydrate moieties stabilize the conformation of the mature glycoprotein (Imperiali and Rickert, 1995; Wang et al., 1996).

Hydroxynitrile lyases catalyze the highly enantioselective addition of HCN to aldehydes or ketones resulting in enantiopure cyanohydrins which are important intermediates in syntheses of chiral building blocks for pharmaceuticals or agrochemicals (Effenberger et al., 2000; Groeger, 2001). Recombinant expression of the *Prunus amygdalus* hydroxynitrile lyase isoenzyme 5 (*Pa*HNL5) in the methylotrophic yeast *Pichia pastoris* yielded overglycosylated secreted protein (Glieder et al., 2003; Weis et al., 2004b). In contrast to enzyme preparations from natural sources, recombinant *Pa*HNL5 exhibited extraordinary stability in a broad range of pH 2.5–6.5 (Glieder et al., 2003). This enzyme property is extremely important, because at low pH the competitive, non-specific chemical reaction is suppressed and the produced cyanohydrins are stable. The molecular foundation of this property is still unclear. One could suspect that the observed overglycosylation of *Pa*HNL5 is responsible for stability at low pH. Complete deglycosylation employing PNGaseF, which cleaves the bond between asparagine and GlcNAc and entirely releases the attached oligosaccharides, was not successful due to the inefficiency of the glycosidase under native conditions (Weis et al., 2004b). In contrast, deglycosylation under native conditions with endoglycosidase H, leaving only one *N*-acetylglucosamine (GlcNAc) on each of the modified Asn residues, worked well, but only moderately influenced stability at low pH (Glieder et al., 2003). Thus, non-glycosylated *Pa*HNL5 cannot be generated by modifying already secreted, glycosylated enzyme. Non-glycosylated *Pa*HNL5 could be produced in bacterial systems as e.g. *E. coli*. However, yields of active, non-glycosylated *Pa*HNL5 after controlled cultivation in a bioreactor were reported to be approximately 50-fold lower than with *P. pastoris* (Ayguen et al., 2004). Thus, in order to get hold of non-glycosylated *Pa*HNL5, either *N*-glycosylation must be inhibited by addition of antibiotics (Onishi et al., 1979), or alternatively individual glycosylation motifs are removed by mutating the coding gene sequence for *Pa*HNL5. *P. pastoris* cultures generally producing glycosylated *Pa*HNL5 were exposed to tunicamycin, a potent inhibitor of *N*-glycosylation. In addition, the individual importance of each glycosylation site for stability at low pH was explored by serine scanning. The substitution of asparagine by serine residues can be expected to be non-destructive to structure (Chou and Fasman, 1978a; Chou and Fasman, 1978b), ensuring the production of correctly folded muteins. The results presented here indicate that one individual *N*-glycosylation site (N118) is essential for high stability of recombinant *Pa*HNL5 at low pH.

2. Materials and methods

2.1. Chemicals, ingredients and materials

Unless otherwise stated explicitly, all chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany) and Becton, Dickinson and Company (Franklin Lakes, NJ, USA), respectively. Sterile water was purchased from Fresenius Kabi (Graz, Austria). Restriction enzymes and endoglycosidase H (EndoH) were purchased from NEB (Beverly, MA, USA). Nitrocellulose filters (0.0025 μm) were obtained from Millipore (Billerica, MA, USA). Concentration columns (VIVASPIN 20 mL PES with a 30 kDa cutoff) were purchased from Sartorius (Goettingen, Germany).

2.2. Strains, plasmids and media

Standard molecular biology procedures were performed according to "Current Protocols in Molecular Biology 2004". *E. coli* XL10 Gold ultracompetent cells (Stratagene, La Jolla, CA, USA) were employed for all *E. coli* cloning and transformation experiments. *P. pastoris* strain GS115 (his4) (Invitrogen, Carlsbad, CA, USA) was employed as expression host. The strain *P. pastoris* GS115 *Pa*HNL5_L1Q (Glieder et al., 2003) expressing the secreted isoenzyme 5 from