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Site-selective chemical protein glycosylation protects from autolysis and proteolytic degradation

David Russell, Neil J. Oldham, Benjamin G. Davis *

Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, UK

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Dedicated to Professor Dr. Hans Kamerling on the occasion of his 65th birthday

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ABSTRACT

Glycosylation is often cited as having a stabilizing effect upon proteins with respect to proteolysis, thermolysis and other forms of degradation. We present here a model study on an autolytic protease that has been chemically glycosylated to produce single glycoforms. The resulting glycosylated enzymes are more stable with respect to their own autolytic degradation and that by other proteases. Kinetic parameters for protease activity with respect to the degradation of small-molecule amide substrate reveal no significant change in inherent activity thereby suggesting that reduced autolysis and proteolysis are a consequence of stabilization, perhaps by steric blockade of cleavage points or alteration of local unfolding kinetics. Variation in glycan identity suggests that greater glycan size leads to greater stabilization.

1. Introduction

Glycans attached to proteins serve various functions. Cell signalling and cell–cell adhesion[1] and regulation[2,3] as well as development[4] and immunity[5] are often dependent on glycosylation. In particular, folding[6] and stability[7] of proteins[8,9] are influenced by the co- and post-translational glycosylation.

In the absence of correct glycosylation, many proteins fold incorrectly. It has been suggested that if proteins fail to fold correctly, the glycans are incorrectly displayed and cannot be processed in trimming steps that lead to expulsion of the protein via the endoplasmic reticulum-associated protein degradation (ERAD) pathway.[10] Therefore, the apparently superfluous glycan trimming steps seen in the endoplasmic reticulum may not simply be a means to glycan structure but steps along a 'quality controlled' protein production line, suggesting a key role for added glycans as indicators of correct protein structure.[11–14] Moreover glycans appear to stabilize tertiary structure[15,16] and also aid folding and transport by protecting proteins from proteolysis.[17]

In nature, glycoproteins occur as a mixture of glycoforms[18] that have the same peptide sequence but differ in site and nature of glycosylation. Few, prescient studies have demonstrated that such glycoforms often display different properties that are modulated by the nature of the glycan.[19] Enhanced proteolytic stability, as a result of glycosylation, has been observed, for example, through the comparison of RNase A, an unglycosylated pancreatic ribonuclease, and RNase B, which bears a single high-mannose oligosaccharide at Asn34.[19–21] Yet these studies have sometimes been limited by the difficulties in obtaining pure sources from nature. Chemical synthesis can provide valuable alternative access to single, pure glycoforms that can allow precise delineation of the effects of attached glycan upon protein function,[22,23] potentially with near-unlimited variation in glycan type and attachment site. Homogenous synthetic glycoproteins have allowed the first systematic determinations of the properties of synthetic glycoforms. Elegant examples have included the synthesis of chemically glycosylated variants of dihydrofolate reductase (DHFR), where mono-glycosylated forms of DHFR showed increased thermolytic stability.[24] Detailed glycan structure–hydrolytic activity relationships towards small-molecule substrates have been obtained for a library of 48 glycosylated forms of a protease.[25] Such glycosylated enzymes have also displayed enhanced catalytic activity in peptide synthesis, and have allowed, for example, the syntheses of D-amino acid-containing dipeptides that were not possible using native unglycosylated enzymes.[26–28] It has also been shown that chemically glycosylated glycoproteins also display enhanced stability under conditions associated with in vivo endosomolytic/lysosomolytic degradation, an advantage that has been exploited in a bipartite drug delivery system employing synthetic glycoprotein.[29]

* Corresponding author. Tel./fax: +44 1865 275652.
E-mail address: Ben.Davis@chem.ox.ac.uk (B.G. Davis).

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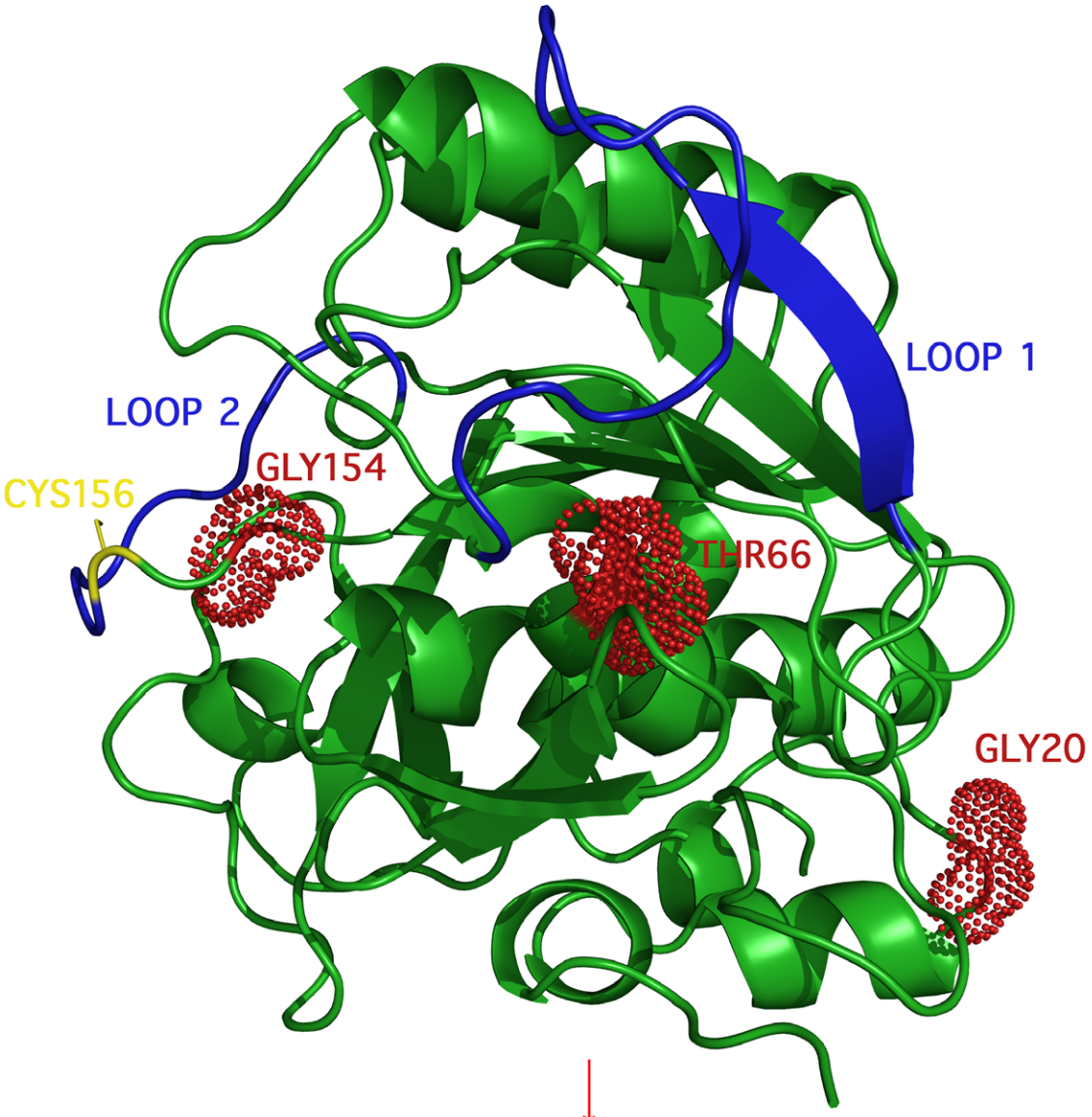


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1     AQSVPWGISR VQAPAAHNRG LTGSGVKVAV LDTGISTHPD LNIRGGASFV
           HHHHH TTHHHHHTTT  S TT EEEE EES     TT   EEEEEE S

51    PGEPSTQDGN GHGTHVAGTI AALNNSIGVL GVAPSAELYA VKVLGASGSG
      TT  S S SS SHHHHHHHHH H  SSSSS    SSTT EEEE EE S TTS

101   SVSSIAQGLE WAGNNGMHVA NLSLGSPSPS ATLEQAVNSA TSRGVLVVAA
       HHHHHHHHH HHHHTT SEE EE   BSS   HHHHHHHHHH HHTT EEEEE

151   SGNCGAGSIS YPARYANAMA VGATDQNNNR ASFSQYGAGL DIVAPGVNVQ
        S   SS   BTTTSTTEEE EEEE TTSSB  TT   STTE EEEEE SSEE

201   STYPGSTYAS LNGTSMATPH VAGAAALVKQ KNPSWSNVQI RNHLKNTATS
      EEETTTEEEE E SHHHHHHH HHHHHHHHHH H TT  HHHH HHHHHHTSB

251   LGSTNLYGSG LVNAEAATR
       S HHHHTT  B  HHHHT
```

Figure 1. The structure (taken from RCSB-PDB structure code 1jea and visualized using PyMol) and primary sequence of SBL-S156C. (a) Putative autolysis loops are shown in blue. Putative cleavage sites identified in this study are shown dotted and in red. Glycosylation site at 156 is shown in yellow. (b) Primary structure is annotated with the following secondary features: H = helix, T = turn, E = extended strand in beta sheet, B = isolated beta bridge. The primary sequence shown here is numbered contiguously according to SBL; all others in this paper, and those in (a), are numbered as per the convention according to subtilisin BPN',[32] which takes into account deletions from SBL to BPN'. Glycosylation site at 156 is shown in yellow. Putative cleavage sites identified in this study are shown with a red arrow.

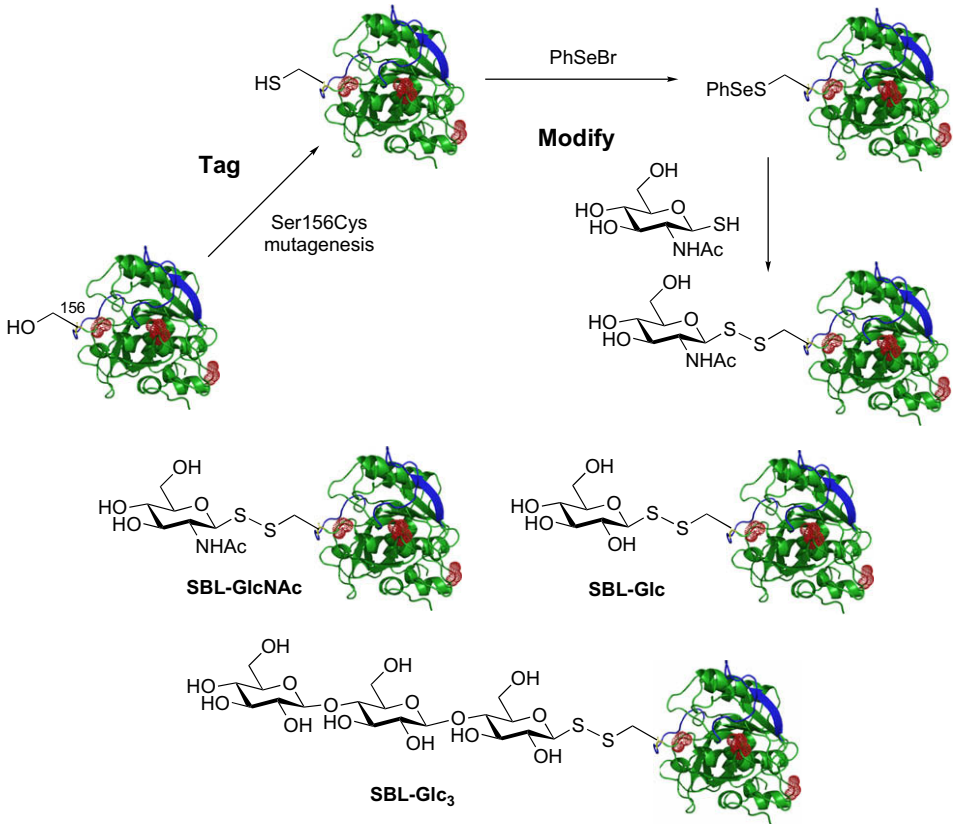
Given the implicated role of glycans in stabilization towards proteolysis,[17] we designed a system that would allow the precise investigation of glycosylation in stabilization towards self-degradation (autolysis) as well as by external proteases. This relied upon two key methods: (i) the tag-modify strategy[30] for site-selective protein glycosylation and (ii) the precise monitoring of enzyme-catalyzed processes using calibrated, quantitative mass spectrometry.[31]

Model protein SBL, the subtilisin (serine endo-peptidase) from *Bacillus lentus*[32,33] was chosen as an example of a class of enzymes, the subtilsins, that have been widely studied in the context of both proteolysis and autolysis[34,35] and engineering[36,37] to improve stability.[38] Excellent structural information is available including those determined by X-ray crystallography to 0.78 Å resolution.[39] SBL (Fig. 1) contains two loops that are similar to those identified previously in a related enzyme, subtilisin BPN', as being primary sites of autolysis.[34] We chose one of these loops (loop 2) as one that we would attempt to influence through site-selective glycosylation at position 156.

2. Results and discussion

A panel of site-selectively glycosylated SBL proteases were constructed using the 'tag-modify' strategy using the thiol side chain in the natural amino acid Cys as a tag and the Glyco-SeS method previously described[40] as a modifying reaction (Scheme 1). Site 156 was chosen as being close to putative autolysis loop 2 that was identified on the basis of homology to a similar loop found in subtilisin BPN', which has been identified as a site of autolysis.[34]

Thus the site-directed Ser→Cys mutant of SBL in which the serine in position 156 was exchanged with a cysteine was constructed. Wild-type SBL contains no cysteine residues, and so mutant SBL-S156C contains only one thiol at position 156 that functions as a 'tag' for chemical glycoconjugation. SBL-S156C was treated with phenylselenyl bromide in acetonitrile buffer (pH 9.5) to prepare the corresponding preactivated selenenylsulfide intermediate. This intermediate was then used to create three different representative glycoproteins containing monosaccharide *N*-acetyl-β-D-glucosamine (in SBL-GlcNAc) or β-D-glucose (in



Scheme 1. The creation of site-selectively glycosylated proteases as pure glycoforms using the tag-modify strategy.

Table 1
Characterization of synthetic glycoproteins

Glycoprotein	Glycosylation reaction[a]			Amidase kinetics[b]			
	Yield (%)	*m/z* calcd	*m/z* found	pH	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
SBL S156C	—	26,714	26,712	8.6	135.6 ± 5	0.89 ± 0.07	152.4
SBL-GlcNAc	>95	26,950	26,948	8.6	133.2 ± 7	0.83 ± 0.07	160.5
SBL-Glc	>95	26,909	26,906	—	nd	nd	nd
SBL-Glc_3	>95	27,233	27,230	—	nd	nd	nd

[a] As determined by ESIMS.
[b] Initial rates method using the small-molecule tetrapeptide succinyl-AAPF-para-nitroanilide as a chromophoric substrate; nd = full kinetic parameters not determined (only SBL-GlcNAc was used in the autolytic study); all enzymes were active towards the substrate.

SBL-Glc), as well as the trisaccharide Glcβ(1,4)Glcβ(1,4)Glcβ (in SBL-Glc_3) using the appropriate glycosyl thiols (Scheme 1). The glycosylated protein was purified on a Sephadex G25 PD10 column, dialyzed against water and then lyophilized. Characterization of the resulting glycosylated proteases (Table 1) confirmed glycosylation identity and activity.

Two enzymes were selected for evaluating the effect of glycosylation upon autolysis activity: unglycosylated SBL-S156C and glycosylated SBL-GlcNAc, which differ only in the presence of a disulfide-linked GlcNAc residue at position 156. Consistent with results from previous studies[41] use of small-molecule tetrapeptide substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide[42] confirmed that modification at position 156 had no intrinsic effect on the inherent catalytic activity of SBL; SBL-S156C and SBL-GlcNAc displayed almost identical Michaelis–Menten parameters (Table 1). Any resulting overall enhanced stability towards autolysis might potentially arise from a combination of two possible factors: a decrease in intrinsic enzymatic activity due to glycosylation or an inherent increase in proteolytic stability of glycosylated protein (becoming a less preferred substrate). The near-identical intrinsic activity (as judged by hydrolysis of suc-AAPF-*p*NA) of glycosylated and unglycosylated variants SBL-S156C and SBL-GlcNAc valuably allowed effects to be attributed to the latter.

The degradation of these enzymes (both autolytically and proteolytically) was assessed using mass spectrometry. While we have previously shown that MS is a powerful technique for determination of kinetic parameters even in complex enzymatic systems that process small molecules,[31] we show here that this method may also be applied to proteins as substrates. MS proved to be a particularly valuable analytical technique. As well as revealing the relative intensities of starting material and products, it also shows the masses of products and fragments produced by breakdown. In the context of autolysis and proteolysis, with reference to the amino acid sequence and knowledge of enzyme specificity, these fractions can be identified, and a better understanding of the enzyme specificity can be obtained. Quantitative analysis necessitated a robust

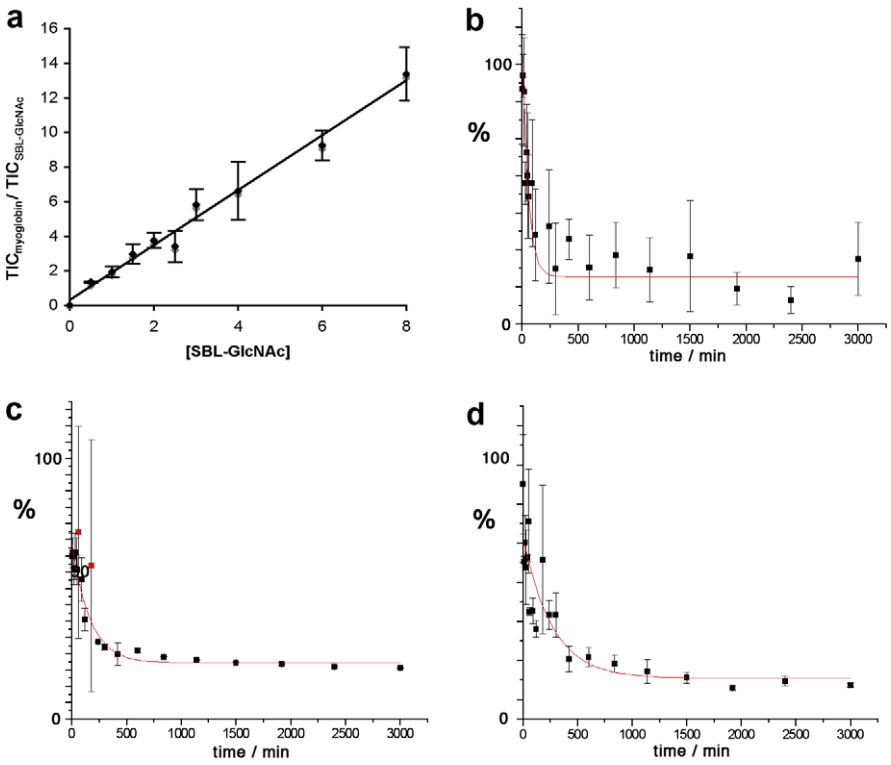


Figure 2. (a) Calibration curve of total ion count (TIC) of myoglobin: TIC SBL-GlcNAc with [SBL-GlcNAc] $R^2 = 0.985$; (b) real-time monitoring of glycoprotein signal of SBL-GlcNAc at 4 mg/mL (% of initial); (c) real-time monitoring of glycoprotein signal of SBL-S156C at 4 mg/mL (% of initial) and (d) real-time monitoring of glycoprotein signal of SBL-GlcNAc at 2.5 mg/mL (percent of initial).

Table 2
Degradation rates under conditions of autolysis and proteolysis

Entry	Glycoprotein	Degradative enzyme	Concentration (mg/mL)	Degradative v_0 (μMs^{-1})	R^2
1	SBL-S156C	SBL-S156C	4.0	0.084	0.97
2	SBL-S156C	SBL-S156C	2.5	0.0049	0.94
3	SBL-S156C	SBL-S156C	1.0	0.0033	0.95
4	SBL-S156C	SBL-S156C	0.5	0.0005	0.95
5	SBL-GlcNAc	SBL-GlcNAc	4.0	0.056	0.89
6	SBL-GlcNAc	SBL-GlcNAc	2.5	0.0015	0.82
7	SBL-GlcNAc	SBL-GlcNAc	1.0	0.0017	0.98
8	SBL-S156C	Pepsin	1.0	2.46	0.96
9	SBL-GlcNAc	Pepsin	1.0	1.21	0.97
10	SBL-Glc	Pepsin	1.0	1.16	0.98
11	SBL-Glc_3	Pepsin	1.0	0.76	0.97

internal standard. Using a constant concentration of horse heart myoglobin (0.04 mg/mL), mass spectra over a range of glycoprotein concentrations were analyzed (Fig. 2).

Analysis of mass intensities using smaller, multiple *m/z* signals was based on maximum entropy (MaxEnt) analysis.[43] The reconstructed MaxEnt spectrum exhibits enhanced resolution and signal-to-noise ratio. The reliability of the result can be assessed by probabilistic methods, which means that a probable error range can be calculated for each mass. The area under each resultant peak in the spectrum is consequently representative of the summed intensities of each component's multiply charged series in the original *m/z* data. Intensities for signals obtained for glycoprotein were compared with those obtained for myoglobin according to several methods: height of peak, total ion count, area under peak, percentage of all peaks or intensity of a single raw charge state. Of these methods, only total ion count gave good correlation with a reasonable coefficient ($R^2 > 0.98$) (Fig. 2a).

Using this method MS allowed real-time monitoring of the depletion of glycoprotein concentration (Fig. 2b–d) in triplicate. Non-linear regression analysis allowed initial rate values to be determined at a variety of concentrations (Table 2).

Analysis of initial rates revealed (Fig. 3a, Table 2) that in all cases glycosylated enzymes were more slowly degraded than their unglycosylated counterparts. Moreover, determination of reaction order using initial rates revealed autolytic orders of 2.10 and 2.15 for SBL-156C and SBL-GlcNAc, respectively. Higher order processes have been observed previously[38,35] and are consistent with various mechanisms of autolysis. Rate-limiting unfolding of key loops (local unfolding) has also been implicated[34,38] in subtilisin systems that are similar to SBL, and it may be that glycosylation alters the rate of these processes too, especially given the proximity of the glycosylation site 156 to cleavage sites (vide infra) and the so-called autolytic loops found in subtilisin BPN' (Fig. 1).

Analysis of mass spectra also allowed putative sites of cleavage to be identified based on the masses of autolysis products. The substrate selectivity of SBL is broad, but the enzyme displays a preference, at least in small substrates with little or no secondary structure, for large uncharged residues at the P1 site and has low activity when this amino acid is a glutamyl residue[44] [the amino acid nearest the N-terminus adjacent to the scissile bond, (from the nomenclature of Schechter and Berger[45])]. However, the primary cleavage sites of SBL are likely to be similar to those of BPN',[34] which do not reflect the inherent substrate selectivity of the enzyme but rather reflect the accessibility of the cleavage site; this in turn reflects the absence of secondary structure, favouring unstructured or unfolded regions. This is also consistent with the notion of local unfolding of key regions prior to proteolysis (vide supra). The so-called autolytic loops of BPN' may be mapped onto SBL (Fig. 1) and are found at residues 44–63 and 156–168. While SBL, with its 269 residues, is highly similar to subtilisin BPN' (62% identical residues), a significant difference between SBL and other subtilisins is a four-residue deletion (161–164) in the S_1 pocket and two single deletions at positions equivalent to residues 35 and 59 in subtilisin BPN'. As a consequence, cleavage site Ser163-Thr164 found in BPN' is absent. Observed fragments for SBL of 8394, 12698 seem likely to correspond to fragments (aa 67–154 and 21–154, respectively.[46] On this basis we tentatively assign the cleavage sites shown in Figure 1. This was further supported by steady-state kinetics for these peaks suggesting sequential buildup of SBL_{21-154} and then of SBL_{67-154}. Regardless, the fact that specific products are produced at early stages of autolysis suggests that the molecule does not completely unfold.

Finally, to further dissect the origin of the apparent increase in stability of precisely glycosylated proteins such as SBL-GlcNAc we investigated whether this stability was extended to resistance to proteolysis by other enzymes. Pepsin A was chosen as a widely used and efficient degradative protease with an alternative pH optimum (pH ~ 2). At this low pH, SBL is effectively inactive, and proteolysis is attributable to pepsin alone. Nonetheless, to ensure the absence of autoproteolysis, all SBL proteins and glycoproteins were irreversibly inhibited with PMSF. Advantageously, pepsin's substrate preference is somewhat similar to that of SBL, cleaving peptide bonds with hydrophobic groups on either side of them. After survey of several conditions, it was found that a 0.3 μg/mL pepsin solution catalyzed sufficient hydrolysis over a useful time course. Initial rates were determined and calculated as for autolysis (Table 2, Fig. 2b). As for autolysis, clear stabilization was observed upon site-selective glycosylation. Based on a greater difference between the initial rates of degradation of $SBL\text{-}Glc_3$ and similar rates for both SBL-GlcNAc and SBL-Glc, we tentatively

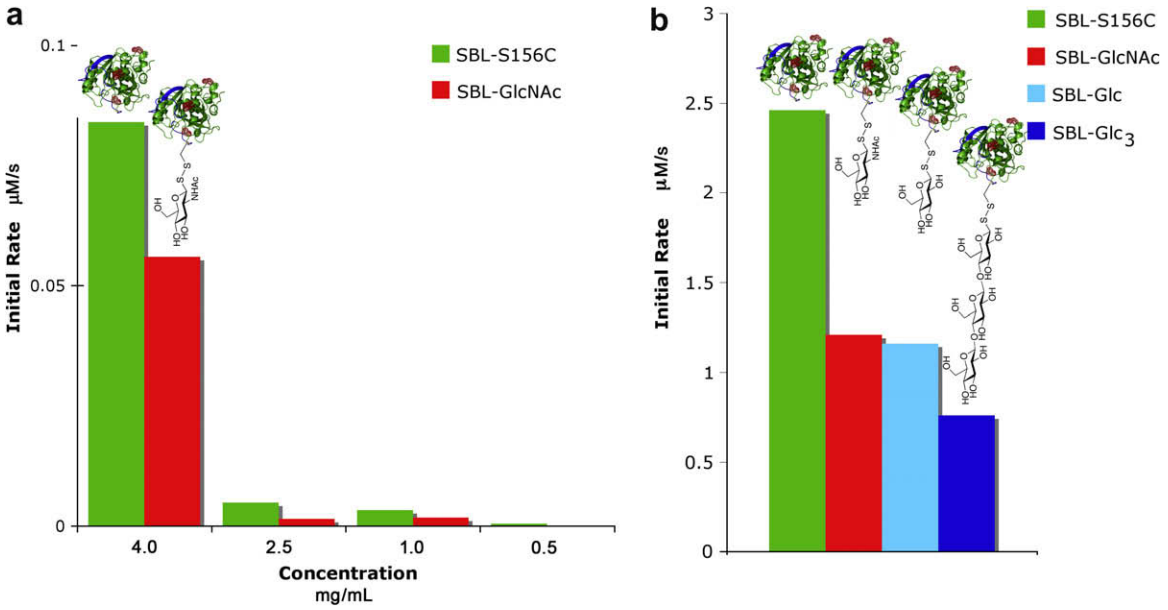


Figure 3. Degradation rates of glycoproteins (a) autolysis of SBL and (b) proteolysis of SBL-glycoproteins by pepsin.

suggest that glycan size is the primary determinant and that larger oligosaccharides may impart more stability.

In conclusion, we have used precise site-selective glycoprotein assembly combined with quantitative MS analysis of product concentration to determine the effects of chemical glycosylation upon stability with respect to proteolysis. Advantageously, we were able to directly monitor product loss rather than indirect monitoring of loss of activity, which could be caused by numerous unrelated factors such as inhibition and/or precipitation. In all cases enhanced stability was observed for glycosylated proteins as compared with direct unglycosylated counterparts. The origin of this stability may be due to several contributing factors such as direct steric blockade of proteolytic sites preventing enzyme–substrate interaction and reduction of the rate of potentially limiting local unfolding events prior to proteolysis.

The enhanced resistance to breakdown apparently conferred by glycosylation, which is observed here at least for site 156 in SBL, may, if general, be of relevance in a variety of applications ranging from stabilization of detergents[35] to enhanced stability of protein therapeutics under conditions of proteolytic degradation. We are currently investigating additional examples in a range of proteins to determine whether this stabilization effect is a general one with implications for the role of biosynthetic glycosylation. It may be that glycosylation sites, which emerge in many organisms subject to a variety of selection pressures, are influenced by proteolytic pressure that may be counteracted by such glycosylation stabilization.

3. Experimental

3.1. General methods

Ligation buffer consisted of 70 mM CHES, 5 mM MES and 2 mM $CaCl_2$, pH 9.5. Water was purified using a Millipore MILLIPAK express 0.22-μm filter. Medicell International Ltd visking dialysis tubing (MWCO 12–14 kDa, size 5 inf. dia. 24/32″-19.0 mm) was prepared by immersion in a 2% $NaHCO_3$ solution containing 1 mM EDTA, and was heated at 80 °C for 30 min, followed by washing with 4 × 1 L of pure water.

3.2. Mass spectrometry

All MS analyses were carried out using a Fisons Instruments VG Platform quadrupole mass spectrometer using an Agilent 1100 Series autoinjector in electrospray-ionization (ESI+) MS mode. The cone voltage was ramped from 30 to 80 V over the mass range. The solvent used was 1:1 acetonitrile–water. MassLynx was used to record the spectra produced. The raw data were first smoothed (peak width: 2.00 Da, number of smooths: 2, smoothing method: mean) and then recalibrated (reference material: horse heart myoglobin, mass range: 600–1800 Da). MaxEnt was used to deconvolute the raw data. MaxEnt1 (resolution: 1.0 Da/channel, damage mode: uniform Gaussian, width at half height: 1 Da, minimum intensity ratios: left 33%, right 33%, number of iterations: 20).

3.3. Site-selective protein glycosylation

The site-selective method was based on the Glyco-SeS protocol previously disclosed.[40] Briefly, specific details are as follows:

SBL-SePh: 60 mg of SBL dissolved in 6 mL of ligation buffer was added to 24 mg of PhSeBr in 200 mL of acetonitrile. This was put on an end-over-end rotator for 1 h, and then loaded onto a Sephadex G-25 PD10 desalting column, and the protein fraction was eluted with pure water.

SBL-Glc: 5 mg of SBL-SePh was dissolved in 1 mL of ligation buffer and added to 0.73 mg glucose thiol (in 2 mg/mL water solution). This solution was put on an end-over-end rotator for 1 h. It was then loaded onto a Sephadex G-25 PD10 desalting column. It was then dialyzed (MWCO 12–14 kDa) against 2 L of pure water for 3 h, with the water being changed every hour. The glycoprotein was then lyophilized.

SBL-GlcNAc: 5 mg of SBL-SePh was dissolved in 1 mL of ligation buffer and added to 0.88 mg of *N*-acetylglucosamine thiol (in 2 mg/mL water solution) put on an end-over-end rotator for 1 h. It was then dialyzed (MWCO 12–14 kDa) against 2 L of pure water for 3 h, with the water being changed every hour. The glycoprotein was then lyophilized.

SBL-Glc$_3$: 4.55 mg of SBL-SePh was dissolved in 1 mL of ligation buffer and added to 1.765 mg of trisaccharide Glcα(1,4)-Glcα(1,4)-Glc thiol (in 2 mg/mL of water solution) put on an end-over-end rotator for 1 h. It was then dialyzed (MWCO 12–14 kDa) against 2 L of pure water for 3 h, with the water being changed every hour. The glycoprotein was then lyophilized.

3.4. Autoproteolysis

Solutions (1 mg/mL) in pure water were made up for each glycoprotein. Glycoprotein solution, 900 μL, was placed into an Eppendorf tube and incubated in a water bath at 23 °C. Aliquots (40 μL) were taken at regular intervals. These were flash frozen and stored at −80 °C. To the 40-μL aliquots were added 40 μL of solvent (MeCN with 4% HCO_2H) and 40 μL of 0.04 mg/mL horse heart myoglobin as internal standard. Aliquots (10 μL) of this mixture were then analyzed directly by MS in triplicate, and the raw spectra were deconvoluted using MaxEnt.

3.5. Amidase activity test

Michaelis–Menten constants were measured at 25 (±0.2) °C by curve fitting (GraFit 3.03) of the initial rate data determined at nine concentrations (0.125–3.0 mM) of succinyl-AAPF-*p*NA substrate in 0.1 M Tris·HCl buffer containing 0.005% Tween 80, 1% DMSO, pH 8.6 (ε_{410} = 8800 M^{-1} cm^{-1}).[47] Absorbance was measured using a Molecular Devices Spectra Max Plus plate reader.

3.6. Pepsin degradation

A 3×10^{-4} mg/mL (0.87 μM) pepsin solution was prepared. To 300 μL of 1 mg/mL glycoprotein in an Eppendorf tube was added 20 μL of the pepsin solution. Aliquots (50 μL) were taken every 15 min. Nine samples were taken over the course of the reaction. The samples were desalted by centrifugation (Micro Centaur, 13000 RPM) on Vivaspin columns (Vivaspin 500 polyethersulfone, MWCO 10,000 Da) with 3 × 300 μL of pure water. The resulting 50-μL samples were then flash frozen using liquid nitrogen and stored at −80 °C. The samples were analyzed by MS.

Acknowledgements

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