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Synthesis of *N*-linked glycopeptides on solid support and their evaluation as protease substrates

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Abstract—A range of glycopeptides containing protease cleavage sites were synthesized on solid support using Fmoc-based solid phase glycopeptide synthesis. The immobilized peptides were studied as substrates for the proteases chymotrypsin and thermolysin. For chymotrypsin, *N*-glycosylation of an Asn residue at the P_2 site appears to reduce hydrolysis whereas glycosylation of the P_1 site does not appear to affect peptide hydrolysis by thermolysin.
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1. Introduction

One of the effects of protein glycosylation has been shown to be protection of the polypeptide backbone from proteolysis by proteases.[1] Proteases are important and abundant enzymes in biological systems, which are often specific for certain polypeptide sequences, characterized as P_1, P_2, P_3,... and P'_1, P'_2, P'_3,..., etc.[2] depending on their position to the scissile bond as shown in Figure 1. Very few studies have been conducted to investigate the susceptibility of glycopeptides to hydrolysis by proteases. Bock et al. have shown that non-natural glycosylation of an aspartic acid residue by lactose and lactitol in the recognition site of the protease savinase can protect soluble glycopeptides from proteolytic cleavage[3] with the effect most pronounced in the P_1 position.

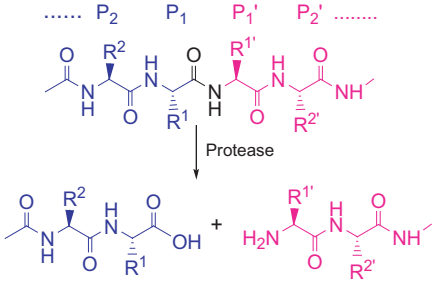

Figure 1. Proteolytic cleavage of peptides and proteins with proteases. The amino acids flanking the hydrolysis site are commonly labeled as P1, P2,... and P1′, P2′,... etc.

Herein, we have looked at naturally occurring glycosylation motifs, in particular *N*-glycan motifs (Asn-X-Ser/Thr) in which the Asn residue is glycosylated by the highly conserved core saccharides (GlcNAc in **1** and chitobiose in **2**) and analogues (Glc in **3** and cellobiose in **4**) shown in Figure 2. These were tested against the commonly used proteases, chymotrypsin from *bovine pancreas* and thermolysin from *Bacillus thermoproteolyticus rokko*.

2. Results and discussion

The core structure of *N*-glycosylation is highly conserved in the majority of higher organisms, both in the peptide and carbohydrate component. Many of these structures will contain the tripeptide codon Asn-X-Ser/Thr, where X can be any amino acid except proline. Furthermore, the *N*-glycan linkage between sugar and polypeptide (Fig. 2), and the first five carbohydrate building blocks (the 'pentasaccharide core' Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc) are universally found throughout nature. The present study has therefore focused on these highly conserved core structures, incorporating at the same time potential proteolysis sites.

In previous work,[4] it was found that on the solid support $PEGA_{1900}$ (polyethylene glycol acryl amide,

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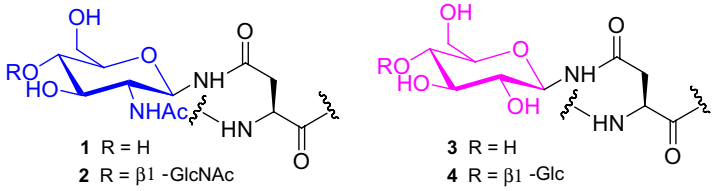
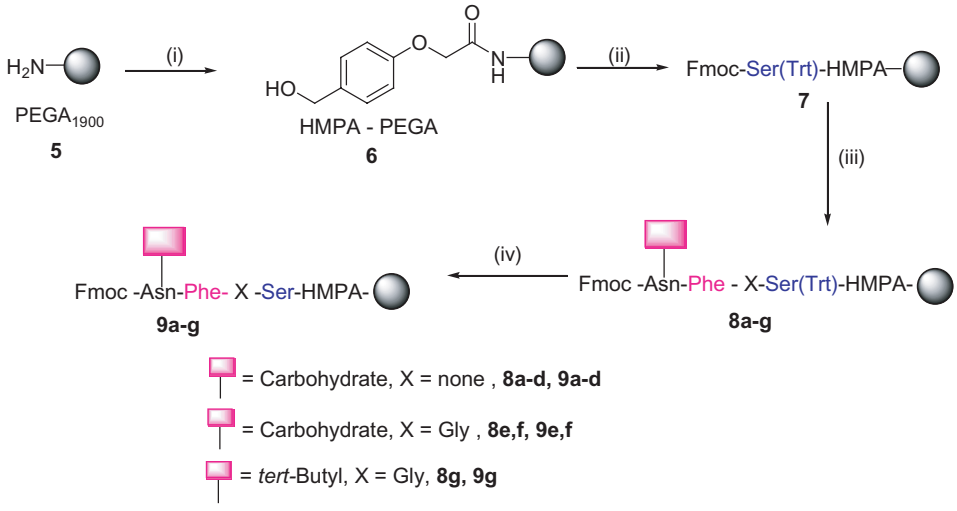


Figure 2. Natural *N*-glycans **1** and **2** and analogues **3** and **4**.

available from Polymer Laboratories, UK) both chymotrypsin and thermolysin have access to all reactive sites and can cleave susceptible peptide bonds on the resin quantitatively. Given that peptides and glycopeptides are often not soluble in aqueous buffers, it was decided to evaluate the substrates on solid phase. Thus, glycopeptides **9a**–**f** and a control peptide **9g** were synthesized on $PEGA_{1900}$, linked to the resin via the Wang linker (HMPA, hydroxymethylphenylacetic acid) (Scheme 1). The unglycosylated peptide **9g** containing a *tert*-butyl ester of aspartic acid was selected as the control peptide sequence because problems with dehydration were encountered when trying to generate peptides with the corresponding natural Asn residue at the glycosylation site on solid support.

In the first step, the HMPA was coupled to amino functionalized $PEGA_{1900}$ resin **5** in the presence of HOBt and DIC in DMF. After 16 h of reaction, the resin **6** was washed thoroughly with DMF, DCM and finally with DMF. Next, Fmoc-Ser(Trt)-OH was coupled by first forming the symmetrical anhydride in the presence of DIC to give **7**. After Fmoc deprotection, the desired protected peptides **8a**–**g** were synthesized using standard Fmoc solid-phase synthesis protocols with Fmoc-L-amino acid/glycoamino acid (4 equiv), TBTU (4 equiv), HOBt (4 equiv) and DIPEA (8 equiv) in DMF for 4 h. In the case of the glycoamino acid couplings, the reaction time was increased to 20 h. The glycoamino acids were synthesized using a short and efficient method recently described in our laboratory.[5] Final deprotection of the serine trityl protecting group was achieved with TFA/TIS/DCM (1:5:94) for 4×5 min to generate compounds **9a**–**g**. The chemical synthesis could be conveniently monitored by hydrolysis of the Wang linker using TFA/H_2O (95:5) and subsequent analysis by LC–MS. This chemical cleavage allowed us to determine the loading of the resin and hence efficiency of subsequent enzymatic hydrolysis. The solid supported peptides **9a**–**g** (0.075 g, 0.001 mmol), were washed thoroughly with buffer and then subjected to enzymatic hydrolysis by incubating with different proteases such as chymotrypsin and thermolysin (2 mg/mL in 0.1 M potassium phosphate buffer, pH 8) for 16 h (Table 1). The hydrolysis mixtures were analyzed by LC–MS.[6]

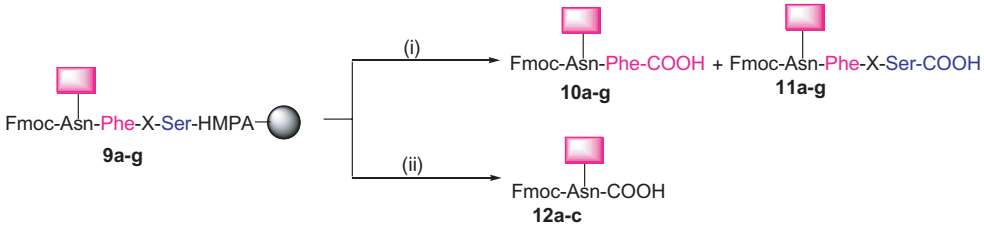
We have recently discovered that the protease chymotrypsin can catalyze the hydrolysis of the ester bond of the Wang linker in peptides such as **9a**–**g** in competition with any peptide bond containing phenylalanine or tyrosine in the P_1 position.[7] Thus two possible products **10** and **11** can be generated by hydrolysis of **9a**–**g** with chymotrypsin. If one assumes that glycosylation at the Asn residue in **9a**–**g** has little influence on cleavage of the Wang linker, formation of **11** is a useful internal standard to look at the susceptibility of the Phe-Ser bond to enzymatic hydrolysis. Such a standard is particularly useful since it is difficult to measure and compare kinetic



Scheme 1. Fmoc-based solid phase synthesis of peptides and glycopeptides **9a**–**g**. The identity of carbohydrate side chains are shown in Table 1. Reagents: (i) HMPA, HOBt, DIC, DMF, rt, overnight. (ii) (a) Fmoc-Ser(Trt)-OH, DIC, DMAP (cat.), DMF, rt, overnight; (b) 20% piperidine/DMF, rt, 1 h. (iii) Fmoc-L-amino acid/glycoamino acid, TBTU, HOBt, DIPEA, DMF, rt, 4 h. (iv) TFA/TIS/DCM (1:5:94), 5 min × 4.

Table 1. Proteolytic hydrolysis results of peptides and *N*-linked glycopeptides

Entry	Glycopeptide/peptide	Enzyme	Product (ratio or yield)
1	Fmoc-Asn(GlcNAc)-Phe-Ser-HMPA-PEGA **9a**	Chymotrypsin	**10a**:**11a** (1:2)
2	Fmoc-Asn(Chitobiose)-Phe-Ser-HMPA-PEGA **9b**	Chymotrypsin	**10b**:**11b** (1:2.5)
3	Fmoc-Asn(Glc)-Phe-Ser-HMPA-PEGA **9c**	Chymotrypsin	**10c**:**11c** (1:2.2)
4	Fmoc-Asn(Cellobiose)-Phe-Ser-HMPA-PEGA **9d**	Chymotrypsin	**10d**:**11d** (1:2.5)
5	Fmoc-Asn(GlcNAc)-Phe-Gly-Ser-HMPA-PEGA **9e**	Chymotrypsin	**10e**:**11e** (1:1.6)
6	Fmoc-Asn(Chitobiose)-Phe-Gly-Ser-HMPA-PEGA **9f**	Chymotrypsin	**10f**:**11f** (1:1.8)
7	Fmoc-Asp(O^{t}Bu)-Phe-Gly-Ser-HMPA-PEGA **9g**	Chymotrypsin	**10g**:**11g** (6:1)
8	Fmoc-Asn(GlcNAc)-Phe-Gly-Ser-HMPA-PEGA **9e**	Thermolysin	**12a** (100%)
9	Fmoc-Asn(Chitobiose)-Phe-Gly-Ser-HMPA-PEGA **9f**	Thermolysin	**12b** (100%)
10	Fmoc-Asp(O^{t}Bu)-Phe-Gly-Ser-HMPA-PEGA **9g**	Thermolysin	**12c** (100%)



Reagents: (i) Chymotrypsin (2 mg/mL of 0.1 M KPi buffer pH 8), rt, 16 h. (ii) Thermolysin (2 mg/mL of 0.1 M KPi buffer pH 8), rt, 16 h. ▭ are carbohydrate side chains as shown in the table.

rates for substrates immobilized to solid support. Previous studies have shown that kinetics can be limited by diffusion of enzyme into the resin.[8]

Analysis of the products of incubating **9a**–**g** with thermolysin and chymotrypsin are shown in Table 1. When the glycosylated peptide **9a** was incubated with chymotrypsin, hydrolysis of the Wang linker was preferred to hydrolysis of the Phe-Ser bond leading to a mixture of **10a** and **11a** in a ratio of 1:2, respectively (entry 1). It was interesting to note that the product **10a** could be isolated, given that in itself it should be a substrate for chymotrypsin. However, we had previously observed that soluble FmocPheAsp and FmocPheGly did not undergo any hydrolysis when incubated with chymotrypsin. A possible explanation might be issues of solubility of the peptide, which could make it inaccessible to enzymatic hydrolysis.

The sugar and the sugar–protein linkage in **9a** present the natural highly conserved core structure. When the next saccharide was added to form chitobiosyl peptide **9b** (entry 2), selectivity for the Wang linker cleavage was not significantly enhanced. Equally, hydrolysis ratios were the same for the unnatural glycopeptides **9c** and **9d** (entries 3 and 4) suggesting that the 2-*N*-acetyl groups of GlcNAc and chitobiose are not involved in stabilization of the linkage towards hydrolysis by chymotrypsin. The ratio of hydrolysis products also remained similar when a glycine residue was inserted into the Phe-Ser sequence (entries 5 and 6), suggesting that cleavage at the Wang linker was not much affected by distant glycosylation. However, when the *N*-glycan site on the peptide in the P_2 position was replaced with the *tert*-butyl ester of aspartic acid **9g** (entry 7), the selectivity was reversed and the Phe-Ser bond was preferentially cleaved to give **10g** and **11g** in a ratio of 6:1. Thus it appears that even a monosaccharide in the P_2 position can inhibit proteolysis by chymotrypsin.

The peptides **9e**–**g** are also potential substrates for the protease thermolysin and were therefore further investigated. Based on the previous research[4] thermolysin would be expected to cleave the Asn-Phe bond but not the Wang linker. Analysis of the incubation products of **9e**–**g** with thermolysin (entries 8–10) appeared to show that glycosylation at the P_1 site did not affect proteolysis giving products **12a**–**c** in quantitative yields.

3. Conclusion

In conclusion, we have shown that the natural *N*-glycans in glycopeptides can protect the peptides from proteolysis. However, our results in combination with those of others show that protection from hydrolysis is highly dependent on the individual protease and on the position of the glycosylation site in respect to the scissile bond.

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