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Articles

N-Linked Glycosylation Prevents Deamidation of Glycopeptide and Glycoprotein

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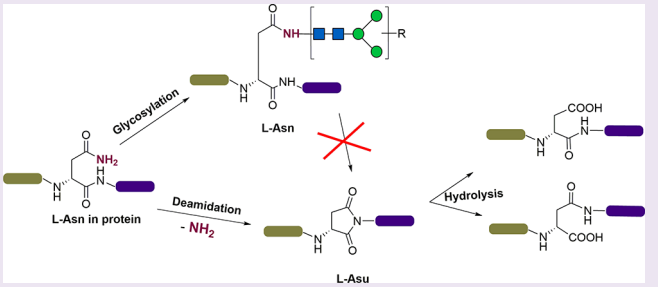
ABSTRACT: Deamidation has been recognized as a common spontaneous pathway of protein degradation and a prevalent concern in the pharmaceutical industry; deamidation caused the reduction of protein/peptide drug efficacy and shelf life in several cases. More importantly, deamidation of physiological proteins is related to several human diseases and considered a "timer" for the diseases. N-linked glycosylation has a variety of significant biological functions, and it interestingly occurs right on the deamidation site—asparagine. It has been perceived that N-glycosylation could prevent deamidation, but experimental support is still lacking for clearly understanding the role of N-glycosylation on deamidation. Our results presented that deamidation is prevented by naturally occurring N-linked glycosylation. Glycopeptides and corresponding nonglycosylated peptides were used to compare their deamidation rates. All the nonglycosylated peptides have different half-lives ranging from one to 20 days, for the corresponding glycosylated peptides; all the results showed that the deamidation reaction was significantly reduced by the introduction of N-linked glycosylation. A glycoprotein, RNase B, also showed a significantly elongated deamidation half-life compared to nonglycosylated protein RNase A. At last, N-linked glycosylation on INGAP-P, a therapeutic peptide, increased the deamidation half-life of INGAP-P as well as its therapeutic potency.



INTRODUCTION

Deamidation is a spontaneous nonenzymatic reaction of peptides and proteins both *in vivo* and *in vitro*.[1−5] Asparagine (Asn)[2,6,7] and glutamine (Gln) are the two amino acids that are susceptible to deamidation, and Asn is much more prone to deamidation than Gln.[8] In the asparaginyl deamidation process, Asn is converted to α-aspartic acid (Asp) and β-Asp (isoAsp) with the loss of an amine group on its side chain through hydrolysis of the succinimide intermediate from different directions (Figure 1).[9,10] During peptide and protein aging, its deamidation site is switched from neutral to negative charge, and a β-linkage in the peptide backbone is formed. The negative charge and the new rotatable bond of β-linkage are able to result in structural and functional disruption.[11−13] For therapeutics, deamidation is one of the most prevalent degradation pathways of proteins and peptides, which is a safety and efficacy concern for protein and peptide therapeutics. For example, growth hormone releasing factor can get a 25−500 times decrease of its potency when deamidation happens.[14] Additionally, asparaginyl deamidation is one of the major contributors to the degradation of monoclonal antibodies,[15,16] and aggregation caused by deamidation is another common concern for protein therapeutics. To our knowledge, it is still difficult to avoid deamidation of protein and peptide therapeutics on a molecular level when they are in manufacturing, manipulation, storage, and especially administration processes.[17,18] As proteins and peptides have grown into major therapeutics in the market in recent years, a practical chemical method is apparently in need to prevent deamidation to increase the stability of peptide and protein pharmaceuticals.[15,18−23]

For human diseases, deamination occurred in certain proteins involved in several human diseases, such as Alzheimer's,[12,24,25] cataracts,[13,26] and Parkinson's.[27] In terms of the physiological system, adverse effects of deamidation which can result in diseases[6,28,29] shall be prevented or controlled through physiological pathways. There are two possible ways for the prevention of deamidation in physiological systems. First, *L*-isoaspartyl *O*-methyltransferase (PIMT) provides a method to repair deamidated proteins.[11,30−32] PIMT can change β-Asp to α-Asp to remove the β-linkage, however, with the unfavorable negative charge left on the peptide backbone. As a result, the repair of

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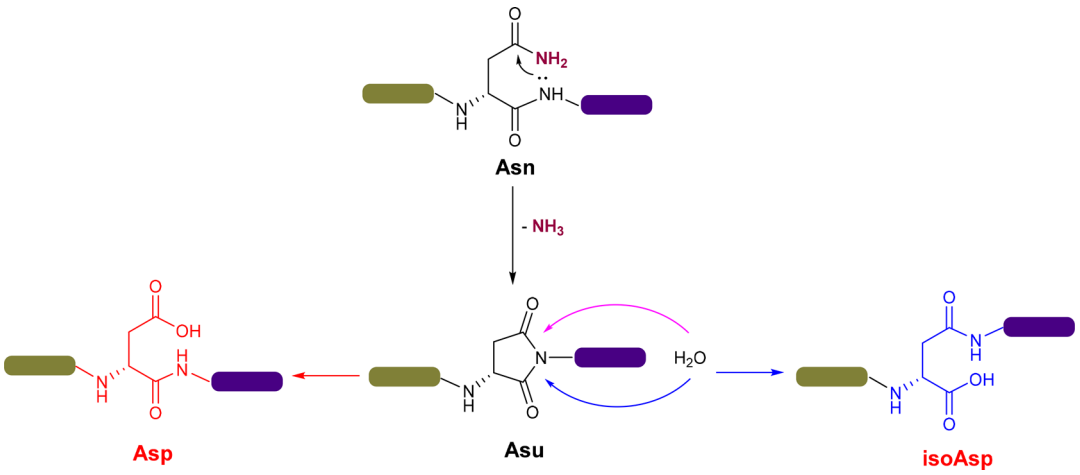

Figure 1. Pathway of deamidation.

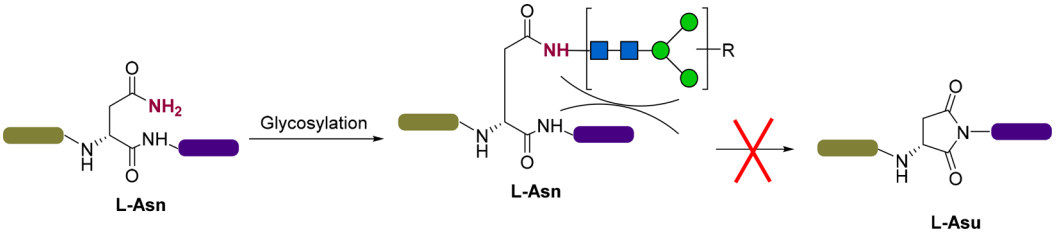

Figure 2. Deamidation Prevented by N-Linked Glycosylation

deamidated protein cannot convert Asp back to Asn, which means it cannot get the protein back to its original Asn type. Second, the conformational structure of protein offers an indirect pathway to reduce the deamidation reaction rate in general.[4] The higher order structure makes the peptide backbone more rigid to form the succinimide intermediate;[33] Asn is thus more stable than that in the flexible backbone. Nonetheless, the deamidation rate is closely related to the primary sequence of proteins instead of the higher order structure. Typically, the primary sequence contributes more to deamidation than three-dimensional structure.[4] N-linked glycosylation is one of the most abundant forms of post translational modification, which bears essential biological functions, such as cell−cell interaction, embryogenesis, facilitating protein folding, and cell signaling.[34,35] N-glycans are mostly and specifically attached to Asn residue in proteins, and N-linked glycosylation is a sequence-dependent process with a consensus amino acid sequence of Asn-Xaa-Ser/Thr known as a sequon.[36,37] The sequon has its own preferences on the $N + 1$ position, where amino acids with lower hindrance are preferred, for example, glycine.[38] Interestingly, amino acid residues with lower steric side chains at the $N + 1$ position tend to make the peptide or protein more susceptible to deamidation, especially when glycine appears at the $N + 1$ position.[4,39] The same reaction site of deamidation and N-linked glycosylation inspired us to believe that N-linked glycosylation performs a function for preventing asparaginyl deamidation to help protect intact protein structures; large N-glycan can serve as a steric hindrance to block the attack from backbone nitrogen to form the succinimide intermediate (Figure 2). Even though it has been believed that N-glycosylation could prevent deamidation,[40] experimental support is still in need to clearly demonstrate this phenomenon. The results of this paper demonstrated that N-linked glycosylation prevented deamidation both in glycoprotein and in glycopeptide. As deamidation has been implicated as a "timer" *in vivo* in several cases,[1,2,41] N-linked glycosylation is herein considered as a "brake" to slow down the "timer" to keep the functions of proteins longer, whereas deamidation is a "timer." Overall, our results showed that N-linked glycosylation has the function of preventing deamidation no matter if it was intentionally designed or coincidentally happened in physiological systems.

In terms of pharmaceuticals, glycosylation has become a principle of therapeutics discovery,[42,43] and N-linked glycosylation has been successfully utilized to improve pharmacokinetic, pharmacodynamic, and biophysical properties of several therapeutic proteins and antibodies.[44−46] Our work herein provides a solution to the problems caused by deamidation to therapeutic proteins and peptides. As a demonstration for the application of this principle in therapeutics, a therapeutic peptide from islet neogenesis associated protein (INGAP-P) was used to demonstrate this strategy; our results showed that preventing deamidation and increasing the drug potency were achieved at the same time. Therefore, we envision that using N-linked glycosylation for preventing deamidation will become a new principle for glycoengineering and chemical modification for increasing the stability of therapeutic proteins and peptides.

RESULTS AND DISCUSSION

Peptides Selection and Synthesis. Five peptides were selected from glycoproteins as our study targets from the Uniprot database. Peptide sequences were selected based on the predication of half-lives.[47,48] The sequence of peptides **1a** and **GP 1** was selected from human α-1-acid glycoprotein 1.[49] The sequence of peptides **2a** and **GP 2** was selected from peptidyl-prolyl cis−trans isomerase FKBP10. The sequence of

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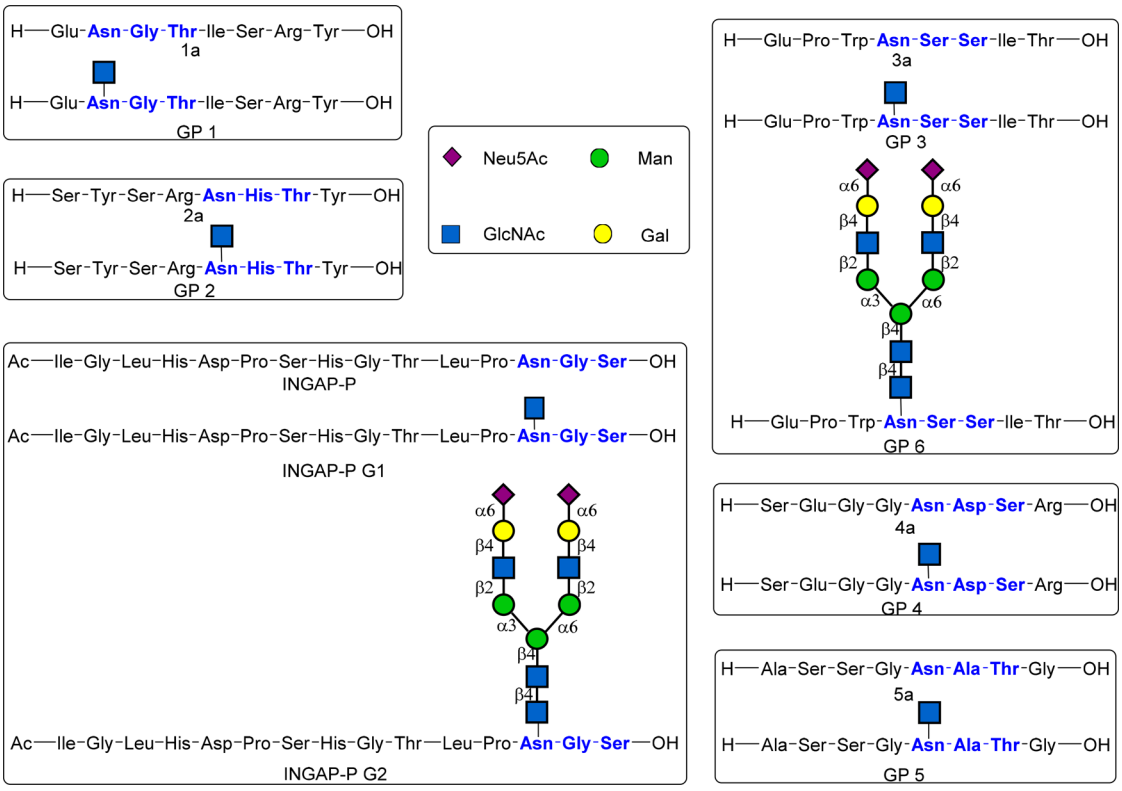
Table 1. Selected Peptides and Corresponding Glycopeptides



peptides **3a**, **GP 3**, and **GP 6** was selected from deoxyribonuclease-2-alpha. The sequence of peptides **4a** and **GP 4** was selected from protein sidekick-1. The sequence of peptides **5a** and **GP 5** was selected from the smoothened homologue. In these peptides, the five sequons are NGT, NAT, NSS, NHT, and NDS. Among them, NGT is the sequon with the lowest steric hindrance at the $N + 1$ position, which should have a faster deamidation rate than the steric amino acids. In NSS, NHT, and NDT sequons, the hydoxy group of serine, the imidazole group of histidine, and the carboxylic acid group of aspartate at the $N + 1$ position have positive catalytic effects on the deamidation reaction.[47,50] The NAT sequon has a more steric effect than NGT and no catalytic effect of the alanine side chain. The corresponding five GlcNAcated peptides were synthesized to test our prediction. On the basis of the proposed mechanism of the deamidation reaction, there are two favored products generated from the deamidation reaction: one is L-aspartate, and the other one is L-isoaspartate. Corresponding aspartate and isoaspartate peptides were also synthesized and used as our standard compounds to facilitate identifying the products of the deamidation reaction of our peptides. Before we test the deamidation reactions of our target peptides, all standard peptides including our target peptides, asparate peptides, isoasparate peptides, and corresponding glycopeptides were used to determine the HPLC methods for the deamidation test. Standard peptides of enzymatic digested RNase A and RNase B and corresponding asparate and isoasparate peptides were synthesized and used to determine HPLC methods for the deamidation tests. INGAP-P and its corresponding glycosylated peptides INGAP-P G1 and INGAP-P G2 were also synthesized for our test (Table 1).

Preventing Deamidation by Glycosylation on NGT Sequon. Peptide **1a** with the NGT sequon has more of a tendency towards deamidation with glycine located next to Asn. In our incubation results, it showed that 32.6% of the undeamidated peptide was left after 48 h incubation (Supplementary Table S1, entry 7). We noticed that the main degradation product of peptide **1a** was a compound which has a longer retention time than either **1b** (Asp) or **1c** (isoAsp) in HPLC results, and the molecular weight was determined by ESI-MS (Supplementary Figure S5A). It is speculated that the degradation product is a diketopiperazine-containing peptide, which was formed by the reaction between the N-terminal amino group and the succinimide intermediate, which was generated by deamidation.[51,52] The deamidation half-life of peptide **1a** was calculated as 31 h with the plotted linear equation (Supplementary Figure S6). In contrast, after 5 days of incubation of the corresponding glycopeptide **GP 1** under the same conditions, the main peak was still the original **GP 1** from HPLC trace (Figure 3B). Five days is 3.8 times longer than the half-life of peptide **1a**, which suggests that the glycosylation prevented deamidation of peptide **1a**. In order to see to what extent the glycosylation can prevent the deamidation of peptide **1a**, we extended the incubation time to 40 days. A small peak was found in the HPLC result of 40 days of incubation, and the speculated diketopiperazine-containing peptide was observed again after the deamidation reaction of **GP 1** (Supplementary Figure S10D,E). **GP 1** was degraded to a large extent within 40 days of incubation (Supplementary Figure S9E). However, both of the two main degraded products were not the deamidation products of peptide **1a** according to ESI-MS results (Supplementary Figure S10B,C). In summary, glycosylation on the NGT sequon of

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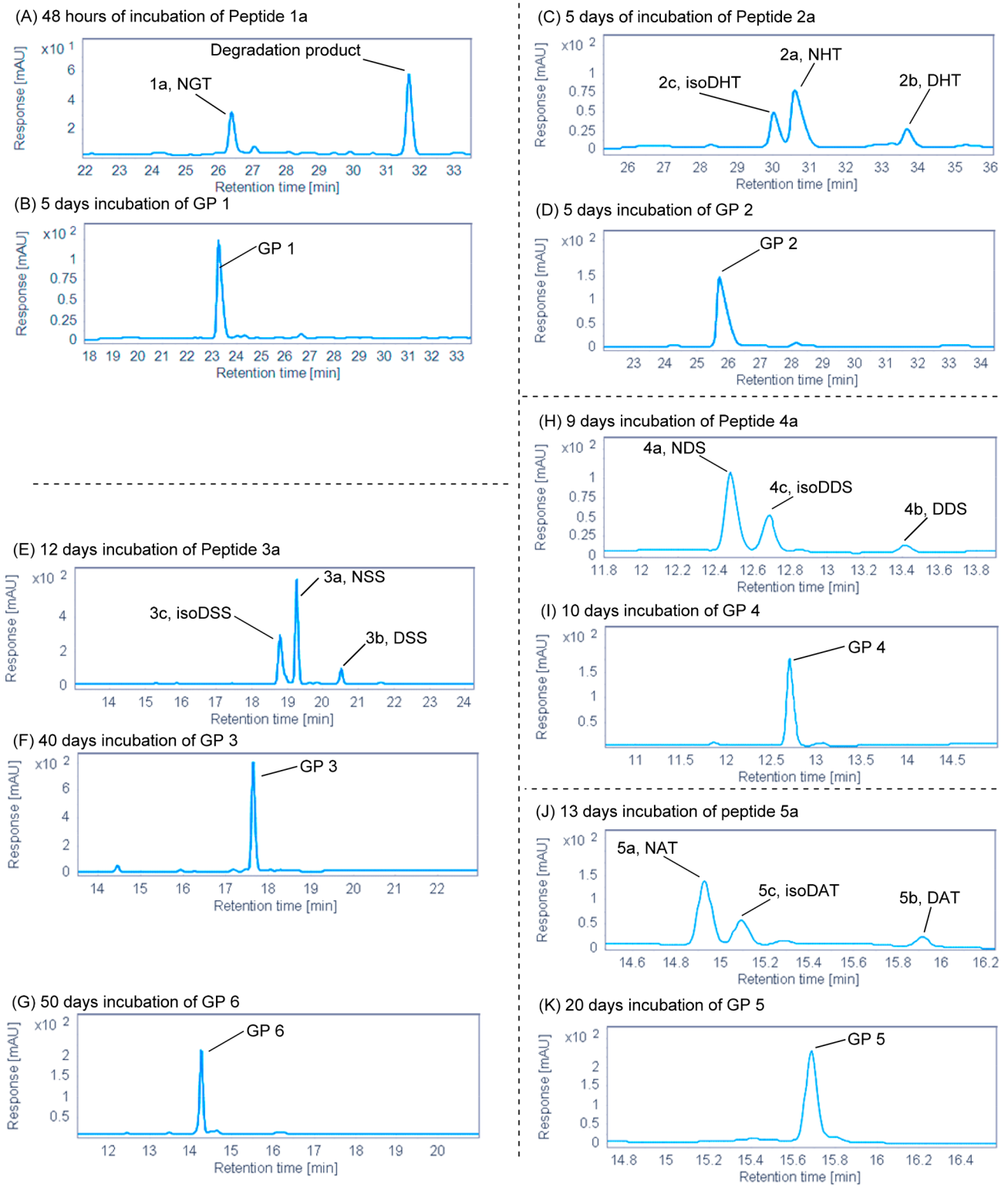

Figure 3. HPLC analysis demonstrating an increase of the stability of glycosylated peptides compared to original peptides through preventing the deamidation reaction: (A) HPLC analysis of 48 h incubation of peptide **1a**; (B) HPLC analysis of 5 days of incubation of glycosylated peptide **1a**−**GP 1**; (C) HPLC analysis of 5 days of incubation of peptide **2a**; (D) HPLC analysis of 5 days of incubation of glycosylated peptide **2a**−**GP 2**; (E) HPLC analysis of 12 days of incubation of peptide **3a**; (F) HPLC analysis of 40 days of incubation of glycosylated peptide **3a**−**GP 3**; (G) HPLC analysis of 50 days of incubation of glycosylated peptide **3a** with natural N-glycan−**GP 6**; (H) HPLC analysis of 9 days of incubation of peptide **4a**; (I) HPLC analysis of 10 days of incubation of glycosylated peptide **4a**−**GP 4**; (J) HPLC analysis of 13 days of incubation of peptide **5a**; (K) HPLC analysis of 20 days of incubation of glycosylated peptide **5a**−**GP 5**.

GP 1 prevented the deamidation reaction of peptide **1a** and makes it more stable than peptide **1a**.

Preventing Deamidation by Glycosylation on NHT Sequon. In peptide **2a**, histidine is located next to Asn and

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provides an intramolecular basic condition to accelerate the reaction rate.[50] Regarding the steric effect of the imidazole ring of histidine, peptide **2a** should have a slower deamidation rate than peptide **1a**. As expected, the deamidation half-life of peptide **2a** was tested and calculated as 6.4 days with the plotted linear equation (Supplementary Figure S15). In contrast, for 5 days of incubation of **GP 2** under the same conditions, the main peak of HPLC was still the starting **GP 2** peak (Figure 3D). Compared to the 5 days of incubation of peptide **2a** (Figure 3C), the deamidation rate of peptide **2a** was reduced. After a 40-days of incubation, the two deamidated products showed up as two little peaks in HPLC, which were identified by our standard compounds and further by ESI-MS/MS (Supplementary Figures S19, S20). Although we can find the deamidated products of **GP 2** after a 40-days of incubation, the deamidation half-life of peptide **2a** was significantly elongated through glycosylation on the NHT sequon.

Preventing Deamidation through Glycosylation on NSS Sequon. For peptide **3a** with the NSS sequon, the hydroxyl methyl group of serine at the $N + 1$ position has a weak catalytic effect on the deamidation reaction, and its side chain has lower steric hidrance than peptide **2a**. It was expected that the deamidation half-life is longer than that of peptide **1a** and longer than that of peptide **2a**. Just as expected, after 12 days of incubation of peptide **3a**, 53.2% of the original peptide **3a** was left (Figure 3E, Supplementary Table S3, entry 6). The deamidation half-life was calculated as 13 days with the plotted linear equation (Supplementary Figure S25). For glycopeptide **GP 3**, the deamidation rate was also significantly slowed down to a very low extent similar to that of **GP 2**, no deamidation products were observed after a 40 day incubation period according to HPLC analysis (Figure 3F). To our delight, the degradation of **GP 3** was also at a very low level. Therefore, it is concluded that the glycosylation on the NSS sequon of glycopeptide **GP 3** significantly prevented the deamidation reaction of peptide **3a** (Supplementary Figure S28).

Preventing Deamidation by Glycosylation on the NDS Sequon. Compared to peptide **3a**, the side chain of aspartate in peptide **4a** with the NDS sequon has higher steric hindrance than serine. Similarly, carboxylic acid also bears a catalytic effect on the deamidation reaction. According to the linear equation of selected different time points, the half-life was calculated as 13.2 days (Supplementary Figure S33). For comparison, the HPLC results of 9 days of incubation of peptide **4a** and 10 days of incubation of **GP 4** showed that the deamidation reaction rate of peptide **4a** was decreased to a large extent due to glycosylation (Figure 3H,I). Although there were new little peaks of 40 days of incubation of **GP 4**, all the new peaks, however, are not deamidated products based on the HPLC results of our standard compounds (Supplementary Figure S37). For glycopeptide **GP 4**, we noticed that it has the same retention time of standard isoAsp peptide **4c**, so it is possible that we did not see one of the deamidation products in HPLC for the incubation of **GP 4**. We observed that the possible deamidated peak showed up in the MS result, but the main MS peak is still the original **GP 4** peak (Supplementary Figure S38B,C). The ratio identified between the isoAsp peptide and Asp peptide of the incubated peptide **4a** is 4.6:1 (determined by HPLC peak areas), which indicates that the deamidation of **GP 4** should have similar results to those of the deamidation of peptide **4a** due to the same succinimide intermediate. However, there was no Asp peak (Supplementary Figure S37A,C) in the HPLC analysis of 40 days of incubation of **GP 4**, which suggested that the amount of deamidated isoAsp product was very little despite observation of the MS peak of isoAsp peptide in the main **GP 4** peak of HPLC. Therefore, we concluded that the deamidation rate of **GP 4** is slowed down by glycosylation on the NDS sequon.

Preventing Deamidation by Glycosylation on NAT Sequon. The methyl group of alanine in peptide **5a** with a NAT sequon is more steric than glycine but less than histidine, serine, and aspartate. Additionally, in contrast to the catalytic side chains, the methyl group cannot provide any catalytic effect on the deamidation reaction, which leaves the steric effect as the only factor affecting the deamidation rate of peptide **5a**. Therefore, comparing the structures of peptide **5a** to those of peptide **1a**, peptide **5a** is predicted to have a longer half-life than peptide **1a**. As expected, using the plotted linear equation, the deamidation half-life of peptide **5a** was calculated as 18.78 days according to our HPLC results (Supplementary Table S5 and Figure S43), the half-life is longer than that of peptide **1a**. Using the same incubation conditions, **GP 5** with 20 days of incubation did not show any deamidation products; in contrast however, peptide **5a** with 13 days had a 37.8% deamidation (Figure 3J,K, Supplementary Table S5, entry 7). It should be noticed that the HPLC peak of Asp standard peptide **5b** overlapped with that of glycopeptide **GP 5** (Supplementary Figure S47A), which made it possible that one of the deamidation products could appear as the same peak as **GP 5**. In order to analyze the components in the HPLC peak of **GP 5** after 40 days of incubation, we collected the incubated **GP 5** peak using HPLC and characterized the components of the peak. The molecular weight of the deamidated product Asp peptide appeared as a small peak together with the main peak of **GP 5** in ESI-MS spectrometry (Supplementary Figure S48B,C). However, there was no isoAsp peptide peak in the HPLC analysis of incubated **GP 5**. In the incubation of peptide **5a**, the ratio between the isoAsp peptide and Asp peptide is 2:1 (determined by HPLC peak areas). The result here is that the amount of isoAsp was too little to observe, which is similar to the case of **GP 4**, which suggested that the ratio of the deamidation product, Asp peptide, in the overlapped peak is very low. Overall, we can conclude that the glycosylation on the NAT sequon reduced the deamidation rate of **GP 5**.

Deamidation Prevented by Natural Biantennary Complex N-Glycan. Since all of the glycopeptides with one GlcNAc have significantly reduced deamidation rates, we speculated that large natural N-glycans should also have a similar function. In order to test our prediction, glycopeptide **GP 6** with natural biantennary complex N-glycan was synthesized to test the function of N-glycan in preventing deamidation reaction. Glycopeptide **GP 6** contains the same amino acid sequence as that of peptide **3a**, and it was incubated for 10, 30, and 50 days. After 50 days of incubation, no deamidation products were detected, and this peptide is almost as stable as **GP 3** based on HPLC results (Figure 3G). Additionally, the biantennary complex N-glycan, especially the two sialic acid residues, was stable enough without any significant degradation during the 50 day course of incubation (Supplementary Figure S50). In brief summary, the experiment demonstrated that the natural N-glycan in **GP 6** also has the same function as GlcNAc of **GP 3** for preventing the deamidation reaction.

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Deamidation Prevention in RNase B through the High-Mannose Type N-Linked Glycosylation. After testing the preventive effect of *N*-glycan in glycopeptides, we switched our focus to glycoprotein RNase B. RNase B and RNase A contain the same amino acid sequence, and the N-linked glycosylation can facilitate the folding process of RNase B, which was found in the process of RNase A folding. The two proteins have similar biological functions and the only difference between them is the high-mannose type *N*-linked glycosylation on the Asn34 residue.[53] Strong basic conditions were adopted for the incubation of RNase A and B to force the deamidation reaction to move forward faster than that in near neutral conditions in the above tests of peptides. RNase A was incubated for 4 days in 0.1 M glycine−NaOH buffer with pH = 9.6 under 78 °C.[54] Deamidated proteins were reported to be studied by a bottom-up method, and trypsin was used as the proteolytic enzyme.[55−57] We applied the method in our study; RNase A and RNase B were digested by trypsin and separated by HPLC. Since trypsin cleavage sites are the C-terminals of lysine and arginine, the NLTK sequence from RNase A and RNase B would be a tryptic peptide theoretically. With the assistance of synthesized isoDLTK and DLTK, we successfully identified the two peptides individually and avoided potential artifacts caused by potential succinimide formation (Supplementary Figure S51−S54). We found that a large portion of Asn34 of RNase A was deamidated according to the MS and MS/MS results compared to that of the synthesized standard compounds (Figure 4a and Supplementary Figure S55). For

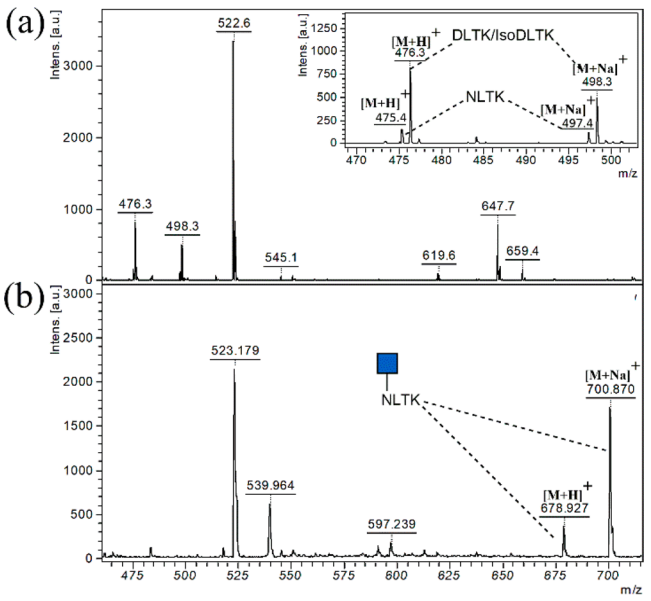

Figure 4. (a) MS identification of Asn34 deamidation of RNase A; (b) MS identification of deamidation prevention through glycosylation of Asn34 in RNase B.

comparison, RNase B was incubated under the same conditions for 4 days and followed by Endo H to remove the high-mannose-type N-glycan except for the first GlcNAc. After trypsin digestion and collection of all HPLC fractions according to the retention time of standard peptides, mass spectrometry was used to analyze the reaction. From the MS and MS/MS results compared to standard peptides (Figure 4b and Supplementary Figure S56), the main peak of this reaction was still GlcNAcated Asn34, and no deamidated peak was found from MS results, which indicated that the high-mannose-type glycan of RNase B prevented the deamidation reaction of RNase B. Thus, it can be concluded that *N*-linked glycosylation prevented the Asn34 deamidation of RNase B.

N-Linked Glycosylation, Single GlcNAc, and Biantennary Complex N-Glycan, Prevents the Deamidation of INGAP-P. After exploration of deamidation prevention through N-linked glycosylation, we tried to apply it to stabilizing a therapeutic peptide, namely, INGAP-P, which has therapeutic effects on both type 1 and 2 diabetes. We noticed that INGAP-P has an NGS sequon at its C-terminal end. After synthesis of INGAP-P, we incubated it under the same incubating conditions as we used above. It is found that 60% undeamidated peptide was left after 40 h of incubation based on analytical HPLC results, and the deamidation half-life was calculated as 55.9 h (Figure 5A, Supplementary Table S6,

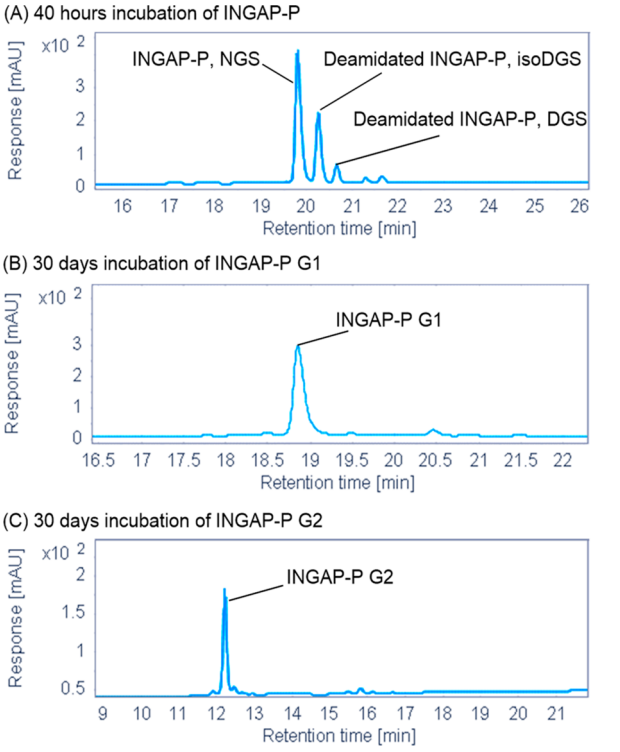

Figure 5. HPLC analysis demonstrating increased stability of glycosylated INGAP-P than that of original INGAP-P through preventing deamidation reaction by glycosylation. INGAP-P G1 and G2 are glycosylated INGAP-P with different N-glycans. (A) HPLC analysis of 40 h incubation of INGAP-P; (B) HPLC analysis of 30 days of incubation of INGAP-P G1; (C) HPLC analysis of 30 days of incubation of INGAP-P G2.

entry 7 and Figure S61). In contrast, glycosylated INGAP-P (INGAP-P G1) is more stable than the original INGAP-P. After 30 days of incubation of INGAP-P G1, almost only one peak of INGAP-P G1 showed up in the HPLC result (Figure 5B). However, we observed two peaks of deamidation products based on HPLC and ESI-MS/MS results after 50 days of incubation (Figure 5C and Supplementary Figure S65). We can conclude that GlcNAc can prevent the deamidation of INGAP-P G1, even though few deamidation products were detected after 50 days of incubation. The other glycosylated INGAP-P, namely, INGAP-P G2, has the same peptide sequence of INGAP-P. After 30 days of incubation under the

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same conditions, the biantennary complex N-glycan of INGAP-P G2 showed a similar ability of preventing the deamidation reaction as well as the GlcNAc of INGAP-P G1 did (Figure 5C). In summary, the deamidation rate of INGAP-P was reduced significantly no matter whther it was through a single GlcNAc or a biantennary complex N-glycan.

Glycosylated INGAP-P Induced Higher Cell Proliferation Compared to Unmodified INGAP-P. We hypothesized that glycosylated INGAP peptides would not have any adverse effects on cell proliferation or would even cause higher cell proliferation in the pancreatic β-cell line RIN-m5F compared to unmodified INGAP-P due to glycosylated INGAP peptides' longer half-life. We used 835 nM peptides to treat cells since it was shown previously[58] that this is the optimal dose for cultured pancreatic β-cells. Our data showed that treating pancreatic β-cells with glycosylated INGAP-P G2 resulted in significantly higher cell proliferation compared to unmodified INGAP-P (a 28.6% increase; $p = 0.01$) (Figure 6).

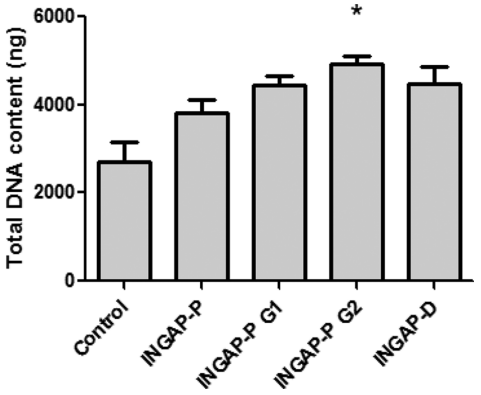

Figure 6. Effect of modified INGAP-P on DNA content in RIN-m5F cells. Cells were treated for 24 h with 835 nM of unmodified or modified INGAP peptides, and total DNA was quantified with a DNA fluorescence assay. $n = 6$ per group. *$p < 0.05$.

We also observed a trend of increasing cell proliferation in response to INGAP-P G1 treatment, although this difference did not reach significance. Treatment with deaminated INGAP-P (INGAP-D) did not reduce cellular proliferation.

Deamidated INGAP-P Significantly Reduced Insulin Secretion. Since various studies showed that INGAP peptides also stimulated insulin secretion in pancreatic β cells,[59,60] we performed an insulin secretion study to examine the effect of modified INGAP peptides on glucose-stimulated insulin secretion in the hamster insulinoma cell line HIT-T15. Our data showed that there was no difference in basal and glucose-stimulated insulin secretion between the control and unmodified INGAP-P treatment. In addition, there was also no significant difference in either basal or glucose-stimulated insulin secretion between unmodified INGAP-P and glycosylated INGAP-P peptides. However, we did observe a significant reduction in basal insulin secretion and a tendency of reduced glucose-stimulated insulin secretion in cells treated with deamidated INGAP-P (INGAP-D; Figure 7). Thus, our data suggest that deamination of INGAP-P has decreased capacity to induce insulin secretion, possibly due to its reduced bioactivity, and this can be prevented by glycosylation of INGAP peptides. Therefore, glycosylated INGAP-P not only increased the stability of INGAP-P, but also increased its potency.

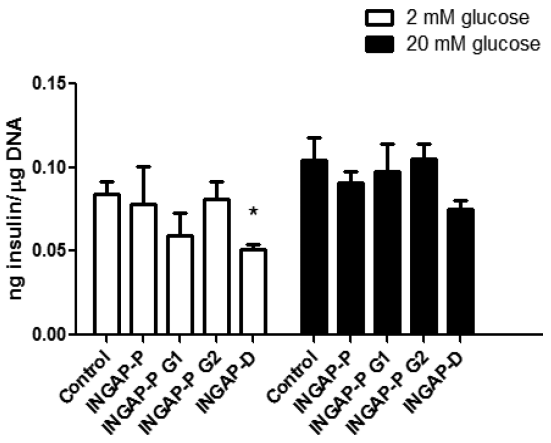

Figure 7. Effect of various INGAP peptides on insulin secretion in HIT-T15 cells. Cells were treated with 835 nM INGAP peptides for 24 h, and then incubated for 1 h with 2 mM or 20 mM glucose. Insulin secretion was quantified by ELISA, and insulin amount were normalized to total DNA amount. $n = 6$ per group. *$p < 0.05$.

CONCLUSIONS

In summary, we have demonstrated the preventing effect of N-linked glycosylation both in glycoprotein and in glycopeptides. Asparaginyl deamidation is the main degradation pathway of all the selected peptides before glycosylation. The deamidation rates of all the glycopeptides were significantly prevented, which showed that N-linked glycosylation prevents deamidation of glycopeptides. The N-glycans of glycoprotein RNase B also showed a similar preventive effect of the deamidation of RNase B. The deamidation reaction was not totally stopped, which means that N-linked glycosylation is to reduce the deamidation rate to a low level instead of fully terminating it, which could bear certain biological significances. Since Asn is considered a "timer" for biological systems, N-linked glycosylation can be used as a modulatory tool by the biological system to adjust the pace of the "timer." This work also provides us an explanation of why glycosylated protein is more stable than nonglycosylated protein. To be mentioned here, N-glycosylation can prevent deamidation significantly, but N-glycosylation is not able to protect N-glycoprotein from other chemical degradations when the incubation time is elongated to a certain time. However, it is still promising that new biotechnologies can be developed to increase the half-life of peptide and protein pharmaceuticals based on the principle of preventing deamidation of glycopeptide and glycoprotein by N-linked glycosylation as deamidation is one of the prevalent degradations of proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00734.

Details of experimental procedures, HPLC results, plots of half-life calculation, and peptide characterization (PDF)

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Notes

The authors declare no competing financial interest.

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