Bioorganic & Medicinal Chemistry 16 (2008) 8366–8372

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Chemoenzymatic synthesis of N-linked neoglycoproteins through a chitinase-catalyzed transglycosylation

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ARTICLE INFO

Article history:
Received 15 July 2008
Revised 17 August 2008
Accepted 20 August 2008
Available online 26 August 2008

Keywords:
Bacillus sp. chitinase
Transglycosylation
Sugar oxazoline
Neoglycoprotein
Chemoenzymatic synthesis

ABSTRACT

A novel application of the *Bacillus* sp. chitinase for the chemoenzymatic synthesis of N-linked neoglycoproteins is described. Three chitinases with different molecular size were purified from the crude chitinase preparation. The purified chitinases were evaluated for their hydrolytic and transglycosylation activity. One chitinase with a molecular size of 100 kDa (Chi100) was identified to be the one with highest transglycosylation/hydrolysis ratio. Chi100 could effectively recognize LacNAc-oxazoline and Manα1,3Glcβ1,4GlcNAc-oxazoline as the donor substrate to glycosylate Asn-linked GlcNAc, while it was unable to recognize Manβ1,4GlcNAc and Man_3GlcNAc-oxazolines as the donor substrates. The chitinase-catalyzed transglycosylation was successfully extended to the remodeling of ribonuclease B to afford neoglycoproteins. Although the yield needs to be optimized, the chitinase-catalyzed transglycosylation provides a potentially useful tool for the synthesis of neoglycoproteins carrying novel N-linked oligosaccharides.

1. Introduction

Chitinases (Ec 3.2.14) are a class of glycosyl hydrolyases that catalyze the hydrolysis of chitin, a linear homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc). These enzymes have been found in a variety of organisms ranging from bacteria to animals. Chitinases belong to two major families of carbohydrate enzymes, family 18 and family 19 (CAZy GH 18 and 19, http://www.cazy.org/), depending on their sequence, structure and mechanism of action.[1] The family 18 chitinases, which include most chitinases from bacteria and fungi, catalyze the hydrolysis of chitin and related substrates via a 'substrate assisted' mechanism involving the formation of an oxazolinium ion intermediate.[2–6]

In addition to their hydrolytic activity, some chitinases were found to possess certain level of transglycosylation activity, that is, the ability to transfer the released oligosaccharide moiety to a suitable acceptor to form a new glycosidic bond. These include chitinase-1 from *Coccidioides immitis*,[7] chitinase A from *Serratia marcescens*,[8] chitinase A from *Vibrio harveyi*,[9] chitinase A1 from *Bacillus circulans*,[10] and chitinases from *Bacillus* sp. (Wako Pure Chemical Inc.). The transglycosylation activity of these chitinases implicates a great potential for the synthesis of oligosaccharides and polysaccharides of chitin origin. For example, Kobayashi and co-workers have successfully applied the chitinases from *Bacillus* sp. for the chemoenzymatic synthesis of artificial chitin and related polysaccharide derivatives, using *N*,*N*′-diacetylchitobiose oxazoline and its modified forms as highly activated monomers for the chitinase-catalyzed polymerization.[11–16] In these studies, a crude chitinase preparation from *Bacillus* sp. was usually used as the catalyst. It was also reported that the *N*-acetyllactosamine oxazoline (LacNAc-oxazoline) could not undergo chitinase-catalyzed self-polymerization because of the blockage of the terminal galactose residue, but it was able to serve as a donor substrate for chitinase-catalyzed glycosylation of a GlcNAcβ-*O*-Me primer to form a trisaccharide moiety, in which the newly formed glycosidic bond was determined to be a β-1,4-linkage.[17] These findings prompted us to explore the potential of the chitinase-catalyzed transglycosylation for glycoprotein synthesis. We and others have previously explored the transglycosylation activity of endo-β-*N*-acetylglucosaminidases for the synthesis of *N*-glycopeptides and glycoproteins, using sugar oxazolines as the donor substrates.[18–26] We reasoned that if the *Bacillus* chitinase could recognize asparagine-linked GlcNAc moiety as an acceptor, then the chitinase-catalyzed transglycosylation would be able to attach an appropriate oligosaccharide moiety to the

Abbreviations: ESI-MS, electrospray ionization-mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; GlcNAcβ-*O*-Me, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; LacNAc, *N*-acetyllactosamine; Man, mannose; MU, 4-methylumbelliferyl; RB, ribonuclease B; RP-HPLC, reverse-phase high-performance liquid chromatography.

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doi:10.1016/j.bmc.2008.08.042

polypeptide to form a new glycopeptide or glycoprotein, when a suitable synthetic sugar oxazoline was used as the donor substrate. However, an initial experiment with crude *Bacillus* sp. chitinase using LacNAc-oxazoline as the donor and a GlcNAc-CD52 as the acceptor[27] failed to obtain any glycopeptide product, mainly due to the hydrolysis of the polypeptide by protease contaminant in the crude chitinase preparation. In this paper, we describe the fractionation of the commercially available crude *Bacillus* sp. chitinase and the examination of the purified chitinase fractions for their transglycosylation activity using an array of synthetic sugar oxazolines as the donor substrates. Our experimental data have shown that the *Bacillus* sp. chitinase was able to glycosylate asparagine-linked GlcNAc in a regio- and stereo-specific manner. Using a suitable sugar oxazoline as the donor substrate and the GlcNAc-containing ribonuclease as a model protein, we have shown that the purified chitinase can attach a specific oligosaccharide moiety to the protein to form a neoglycoprotein.

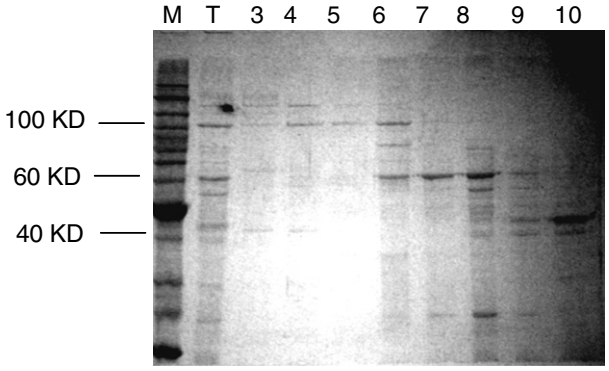


Figure 1. Purification of chitinase from *Bacillus* sp. through size exclusion chromatography. Fractions of the highest chitinase activity were analyzed on a 10% SDS–PAGE electrophoresis. M, protein marker, T, chitinase mixtures before purification. Lane 5, Chi100; lane 7, Chi60; lane 10, Chi40.

2. Results and discussion

2.1. Fractionation of the commercially available *Bacillus* sp. chitinases

The commercially available crude chitinase preparation from *Bacillus* sp. (Wako Chemicals Co.) is a mixture of chitinases and has residual protease activity. Although large amount of the crude chitinase preparation (up to 10% of the substrate weight) was usually used for the enzymatic polymerization of synthetic disaccharide oxazolines,[11–16] it is expected that the application of the crude chitinase to GlcNAc-peptide/protein substrates would lead to degradation of the polypeptides by the contaminant proteases. Indeed, incubation of the model GlcNAc-CD52 peptide[27] [Gly-Gln-Asn(GlcNAc)-Asp-Thr-Ser-Gln-Thr-Ser-Ser-Pro-Ser-NH_2] with the crude chitinases resulted in quick degradation of the peptide, as monitored by HPLC (data not shown). To obtain pure chitinases, we have fractionated the crude chitinase preparation by a high-resolution size exclusion chromatography (Superdex 200). The protein fractions were assayed for chitinolytic activity. Three major protein fractions that have chitinase activity were enriched, which are about 100 kDa, 60 kDa and 40 kDa in size, respectively (Fig. 1, lanes 5, 7 and 10). We named those three chitinases as Chi100, Chi60 and Chi40, respectively. No protease activity was detected in all these three fractions. Therefore, the three chitinases isolated will be suitable for the test for glycopeptide synthesis. Unfortunately, an attempt to identify those chitinases through tandem mass spectrometry failed, as the MS data yielded no positive match(s) to existing protein/peptide sequences in the databases.

2.2. Hydrolytic and transglycosylation activity of individual chitinases

To determine which chitinase is the more suitable candidate for the synthesis of neoglycoproteins, we determined the hydrolytic activity, as well as the transglycosylation activity of the purified chitinases. The rate of hydrolysis was measured using $(GlcNAc)_2$-MU (**1**) as a substrate, which gave the fluorescent 4-methylumbel-

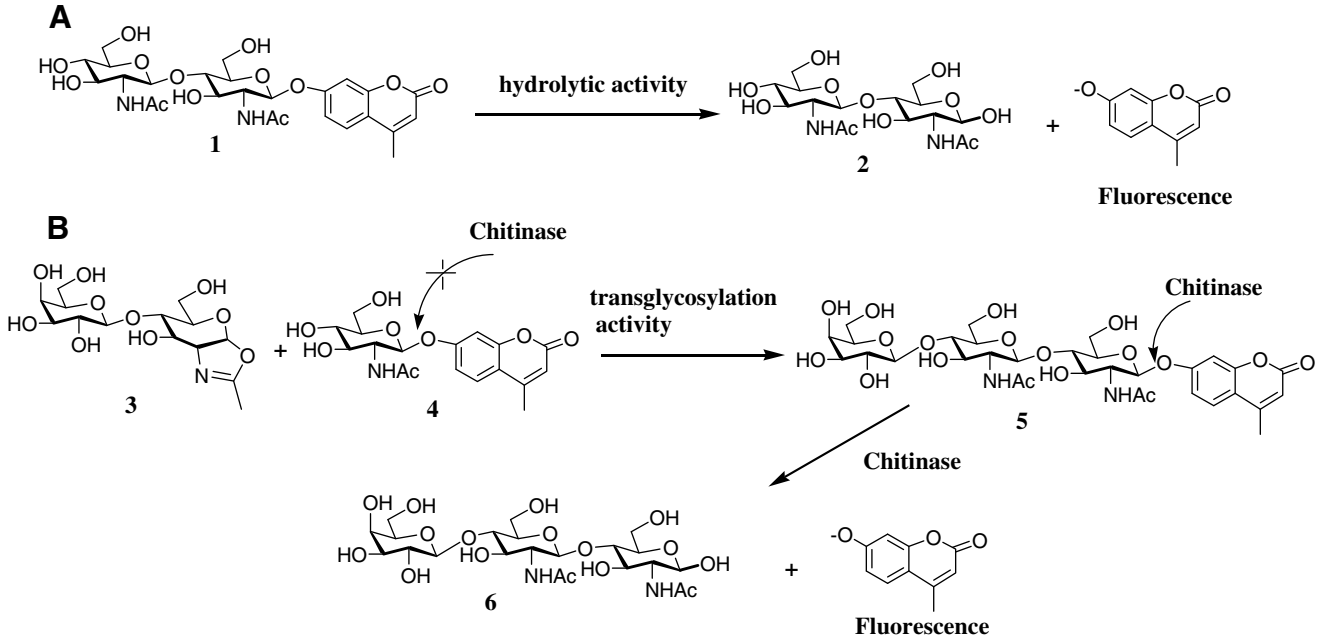


Figure 2. Fluorogenic assays for the chitinases activity. The hydrolytic activity was assayed by using the fluorogenic substrate $(GlcNAc)_2$-MU (**1**) (A) and the transglycosylation activity was determined by a coupled assay using GlcNAc-MU (**4**) as the acceptor (B).

liferone upon hydrolysis (Fig. 2A). It was found that all three chitinases purified could readily hydrolyze the fluorescent substrate, and the Chi100 was the chitinase with the lowest hydrolytic activity. While the Chi60 and Chi40 were found to have a comparable hydrolytic activity, the Chi100 has only about 60% activity of that of the other two chitinases (Fig. 3A).

We then examined the transglycosylation activity of the three chitinases. We designed a simple fluorometric assay using LacNAc-oxazoline **3** as the glycosyl donor and GlcNAc-MU (**4**) as the acceptor (Fig. 2B). This was similar to the coupled assay that we have previously reported for detecting the transglycosylation activity of endo-β-*N*-acetylglucosaminidases.[28] This assay was based on our assumption that the monosaccharide derivative GlcNAc-MU (**4**) would not be hydrolyzed by the *Bacillus* chitinases, in analogy to most chitinases that require at least an additional GlcNAc unit at the −2 position for recognition. Chitinase-catalyzed transglycosylation would lead to the formation of a trisaccharide derivative, Gal(GlcNAc)$_2$-MU (**5**), which had the required (GlcNAc)$_2$-MU recognition motif and was supposed to become a substrate for the chitinase. Thus *in situ* hydrolysis of the newly formed Gal(GlcNAc)$_2$-MU (**5**) would give the fluorescing MU, reflecting the transglycosylation activity (Fig. 2B). It was shown that incubation of the three chitinases with GlcNAc-MU (**4**) alone gave only marginal fluorescence, confirming that the *Bacillus* chitinases could not hydrolyze the monosaccharide derivative GlcNAc-MU (**4**) (Fig. 3B, the control). However, in the presence of the donor substrate LacNAc-oxazoline (**3**), all the three chitinases generated fluorescence, suggesting that the three purified chitinases all possess some transglycosylation activity (Fig. 3B). In comparison, the Chi100 and Chi60 have roughly the same transglycosylation activity, but the Chi40 has much less transglycosylation activity than the other two. The extent of transglycosylation of these chitinases was also determined by measuring the overall fluorescence yield after the reaction was complete. Chi100 and Chi60 yielded comparable amount of fluorescence while Chi40 yielded only 50% of that quantum. These results indicated that the transglycosylation activity of Chi40 was lower than that of Chi100 and Chi60. Taken together, these experiments indicate that the Chi100 chitinase has relatively low hydrolytic activity while possesses a relatively high transglycosylation activity, making it the enzyme of choice for our synthetic purpose.

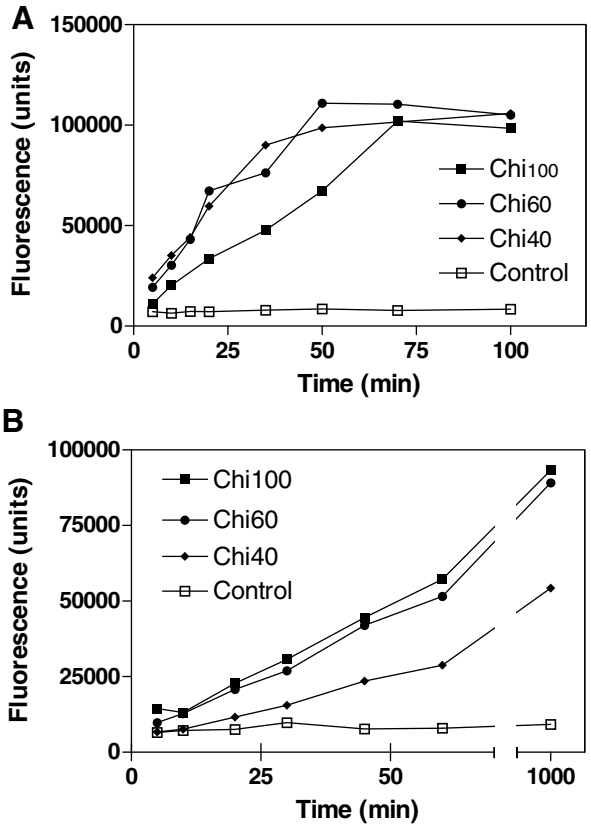
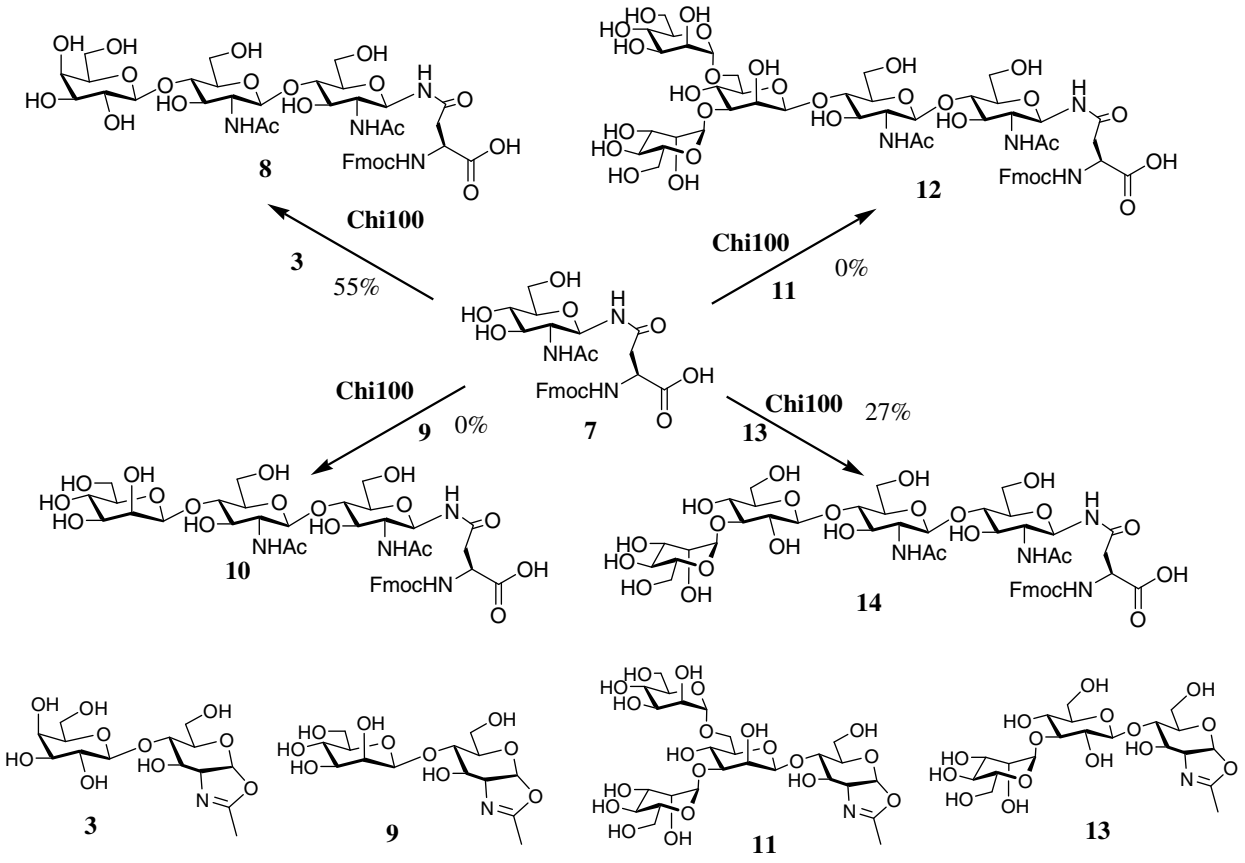


Figure 3. Assay for the hydrolysis (A) and transglycosylation activity (B) of Chi100 (■), Chi60 (●), Chi40 (♦) and controls (□). For hydrolysis (A), the reaction was carried out at 37 °C using (GlcNAc)$_2$-MU (**1**) (0.1 mM) as the sole substrate for the chitinases (0.5 μg). The amounts of the releases MU were determined by fluorescence spectrometry (Arbitrary Unit), and (GlcNAc)$_2$-MU without the addition of the enzymes was used as a control. For transglycosylation (B), the reaction was carried out using LacNAc-oxazoline (**3**) (0.4 mM) as the glycosyl donor and GlcNAc-MU (**4**) (1 mM) as the acceptor, which were incubated with the respective chitinase (0.5 μg) in a phosphate buffer (50 mM, pH 6.5) at 30 °C. The product Gal-(GlcNAc)$_2$MU (**5**), once formed, would be quickly hydrolyzed by the same chitinase and the released MU was then determined by fluorescence (Arbitrary Unit). GlcNAc-MU with enzymes only was used as a control.

2.3. Chitinase-catalyzed transglycosylation of Asn-linked GlcNAc

To examine whether the *Bacillus* chitinase is able to efficiently recognize Asn-linked GlcNAc moiety as an acceptor in transglycosylation, we first carried out a model reaction with an Fmoc-protected Asn-linked GlcNAc (**7**) as the acceptor and LacNAc-oxazoline (**3**) as the donor substrate. The enzymatic reaction was monitored by reverse phase HPLC. While there was no ligation occurred in the absence of the enzyme, the transglycosylation between glycosyl donor **3** and acceptor **7** (molar ratio, 12:1) proceeded quickly with Chi100 to give the trisaccharide derivative **8** with a yield of 55% (Scheme 1). We have also observed that the relatively moderate transglycosylation yield, even when over 10-fold oxazoline donor substrate was used, was not due to the hydrolysis of the transglycosylation product, but was due to the competing hydrolysis of the oxazoline substrate by the enzyme. It was found that incubation of the oxazoline **3** with the chitinase in the absence of the acceptor resulted in graduate hydrolysis of the oxazoline, as monitored by Dionex HPAEC analysis (data not shown). The identity of this product was confirmed by ESI-MS and ^{1}H NMR analysis. The observed molecular mass (923.14 Da) of the product matched well with the calculated mass (Exact mass, 923.33 Da), suggesting that it was the adduct of the disaccharide oxazoline and the Asn-linked GlcNAc acceptor. On the other hand, the β1,4-glycosidic linkage for the newly formed glycosidic bond was verified by NMR analysis. The ^{1}H NMR of the trisaccharide derivative showed that the H-1′ proton appeared at δ4.38 as a doublet with a large coupling constant ($J_{1',2'}$ = 7.5 Hz). This result is consistent with previous report on the stereochemistry of the chitinase-catalyzed transglycosylation when GlcNAc-β-OMe was used as the substrate.[17] Our results clearly indicate that the Asn-linked GlcNAc is a good acceptor for the transglycosylation catalyzed by the chitinase.

Next we tested several other oligosaccharide oxaozlines to examine the donor substrate specificity (Scheme 1). When Manβ1,4GlcNAc-oxazoline (**9**)[18] was incubated with acceptor (**7**) in the presence of Chi100, no formation of the trisaccharide derivative (**11**) was observed, as monitored by HPLC. This result was in contrast to the report for another chitinase-catalyzed transglycosylation, in which the chitinase A1 from *Bacillus circulans* WL-12 was shown to be able to take Manβ1,4GlcNAc-oxazoline (**9**) as substrate, albeit the low transglycosylation yield.[29] These results suggest that the two chitinases, Chi100 from *Bacillus* sp. and A1 from *Bacillus circulans* WL-12, might have distinct substrate specificity. That is, the chitinase A1 from *Bacillus circulans* WL-12 could accommodate the replacement of the GlcNAc at the subsite −2 position by a mannose residue, but the newly isolated chitinase Chi100 from *Bacillus* sp. apparently could not. Similarly, the incubation of a larger oxazoline



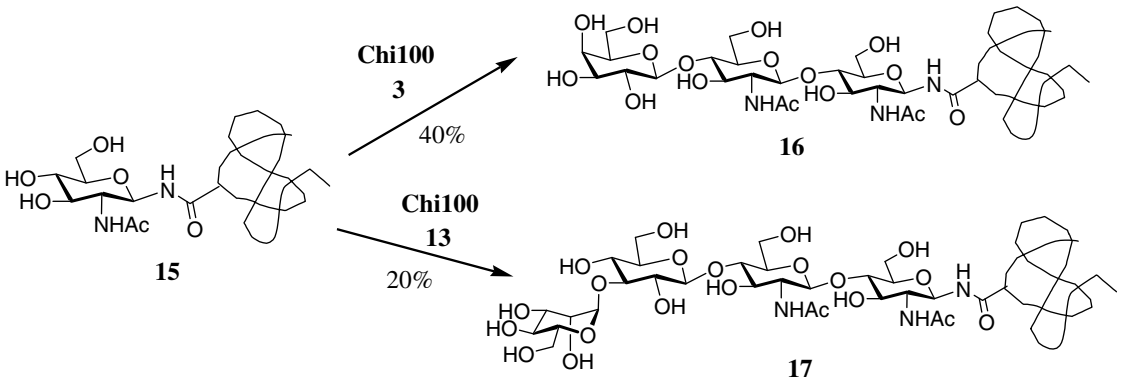
Scheme 1. GlcNAc-Asn as the acceptor for chitinase-catalyzed transglycosylation. Reaction and conditions: The enzymatic reaction was performed in a phosphate buffer (50 mM, pH 6.5) at 30 °C. The reaction was monitored by HPLC.

Man_3GlcNAc-oxazoline (**11**) with Chi100 and acceptor (**7**) did not yield the oligosaccharide **12**, either. The results suggest that the Chi100 does not recognize a mannose residue at the subsite −2 position of the oxazoline substrates. On the other hand, another disaccharide oxazoline, the Glcβ1,4GlcNAc-oxazoline in which the GlcNAc at the −2 subsite was replaced with a glucose residue, was shown to be a substrate for crude *Bacillus* sp. chitinase for the enzymatic polymerization,[14] suggesting that *Bacillus* sp. chitinases could accommodate a glucose residue at the subsite −2 position. Thus we next examined a trisaccharide oxazoline **13**, Manα1,3Glcβ1,4GlcNAc-oxazoline, as a potential substrate for chitinase-catalyzed transglycosylation. Oxazoline **13** has been previously shown to be a substrate of endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis*.[23] The attachment of a mannose residue at the terminal Glc residue was intent to block the self-polymerization of the oxazoline under the chitinase catalysis, as the terminal glucose residue could serve as an acceptor. Thus we synthesized the oxazoline **13** following the reported procedure,[23] and tested its activity with Chi100. It was found that the reaction of oxazoline (**13**) and acceptor (**7**) (donor: acceptor 12:1) in the presence of Chi100 gave the tetrasaccharide derivative (**14**) in 27% yield (Scheme 1). Again, the product was characterized by ESI-MS and NMR analysis to be the expected structure.

Taken together, these results suggest that the chitinase Chi100 could tolerate the replacement of the second GlcNAc residue (subsite −2 position) in the disaccharide oxazoline by a galactose or a glucose residue, but does not recognize a mannose residue at the subsite −2 position. In comparison, galactose and glucose have the 2-hydroxyl group at the equatorial position as in the case of the 2-acetamido group in the GlcNAc residue. Thus when the subsite −2 GlcNAc was substituted by a galactose or a glucose, the 2-hydroxyl group in the galactose or glucose moiety might be able to play a similar role as the 2-acetamido group of the GlcNAc in the chitinase binding and recognition, such as its involvement in hydrogen-bonding. In contrast, the 2-hydroxyl group in the mannose moiety is in the axial configuration. The axial-oriented 2-hydroxyl group in the mannose moiety might not be involved in the similar H-bonding, or it might conflict with other residues in the enzyme sites, thus abrogating the substrate's binding to the enzyme when installed in the oxazoline substrate. On the other hand, the fact that the trisaccharide oxazoline Manα1,3Glcβ1,4GlcNAc-oxazoline (**13**) could serve as a substrate for the chitinase implicates that chitinase Chi100 could tolerate 'unnatural' modification beyond the subsite −2 position in the substrate, as the attachment of a mannose residue at the non-reducing terminal glucose residue in **13** did not abrogate its substrate activity toward the chitinase. It should be noted that a more detailed analysis of the binding and recognition mode would rely on the structural information of the *Bacillus* sp. chitinases, which is presently lacking.

2.4. Synthesis of N-linked neoglycoproteins through the chitinase-catalyzed transglycosylation

After showing that the chitinase Chi100 could recognize asparagine-linked GlcNAc residue as an acceptor for transglycosylation, we have next chosen the bovine ribonuclease B as a target to demonstrate the feasibility of the chitinase-catalyzed transglycosylation for neoglycoprotein synthesis. Bovine ribonuclease B (RB) is a small glycoprotein consisting of 124 amino acids with a single glycosylation site at Asn-34. Native bovine RB is glycosylated with



Scheme 2. Synthesis of neoglycoproteins by the chitinase-catalyzed transglycosylation. Reaction and conditions: The enzymatic reaction was performed in a phosphate buffer (50 mM, pH 6.5) at 30 °C. The reaction was monitored by HPLC.

heterogeneous high-mannose type glycans ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ in size. We and others have used RB as a model glycoprotein to examine new chemoenzymatic approaches for glycoprotein synthesis.[21,30,31] The native ribonuclease B was first treated with Endo-H to remove the *N*-glycans ($Man_{5-9}GlcNAc$) at Asn-34 to obtain the GlcNAc-RB (**15**), leaving only the innermost GlcNAc attached to the Asn-34. The GlcNAc-RB (**15)** was then incubated with LacNAc-oxazoline (**3)** in the presence of Chi100 in a phosphate buffer (pH 6.5) at 30 °C (Scheme 2, molar ratio 60:1). The translycosylation of the GlcNAc-RB (**15**) occurred to give the transglycosylation product **16** in ca. 40% yield, as estimated by ESI-MS (Fig. 4A). However, an attempt to separate the transglycosylation product $Gal(GlcNAc)_2$-RB (**16)** from the starting material GlcNAc-RB (**15)** by reverse phase HPLC was unsuccessful, which have about the same retention time under various HPLC conditions. When Manα-1,3-Glcβ-1,4-GlcNAc-oxazoline (**13**) (in 60-fold molar excess) was used as the donor substrate, the chitinase (Chi100) was able to attach the oligosaccharide moiety to GlcNAc-RB (**15**) to form the neoglycoprotein Manα1,3Glcβ1,4GlcNAcβ1,4GlcNAc-RB (**17**). In this case, the neoglycoprotein could be partially separated under RP-HPLC and the product was obtained in 20% yield. Its ESI-MS profile was shown in Figure 4B and the HPLC profile of the transglycosylation was shown in Figure 4C. Although the yield needs to be optimized, the experimental data clearly established that the purified *Bacillus* chitinase (Chi100) was useful for the chemoenzymatic synthesis of neoglycoproteins carrying novel oligosaccharides. To improve the overall efficiency of the chitinase-based chemoenzymatic glycoprotein synthesis, future work should focus on mechanistic studies and on searching for new chitinases and related mutants with enhanced transglycosylation activity.

3. Conclusion

Three chitinases were purified from the crude *Bacillus sp.* chitinase preparation and were evaluated for their hydrolytic and glyco-transferring activities. The chitinase Chi100 was found to have the highest ratio of transglycosylation versus hydrolytic activity. The Chi100 was able to glycosylate Asn-linked GlcNAc when an appropriate sugar oxazoline was used as the donor substrate. The chitinase was also successfully used for the transglycosylation of ribonuclease B under native conditions without the need to denature the protein, which suggested that this chitinase could be useful for remodeling of glycoproteins under native conditions. This study demonstrated the feasibility of chitinase as a new class of enzymes for glycoprotein synthesis, providing yet another useful tool for the preparation of homogenous neoglycoproteins carrying novel oligosaccharides.

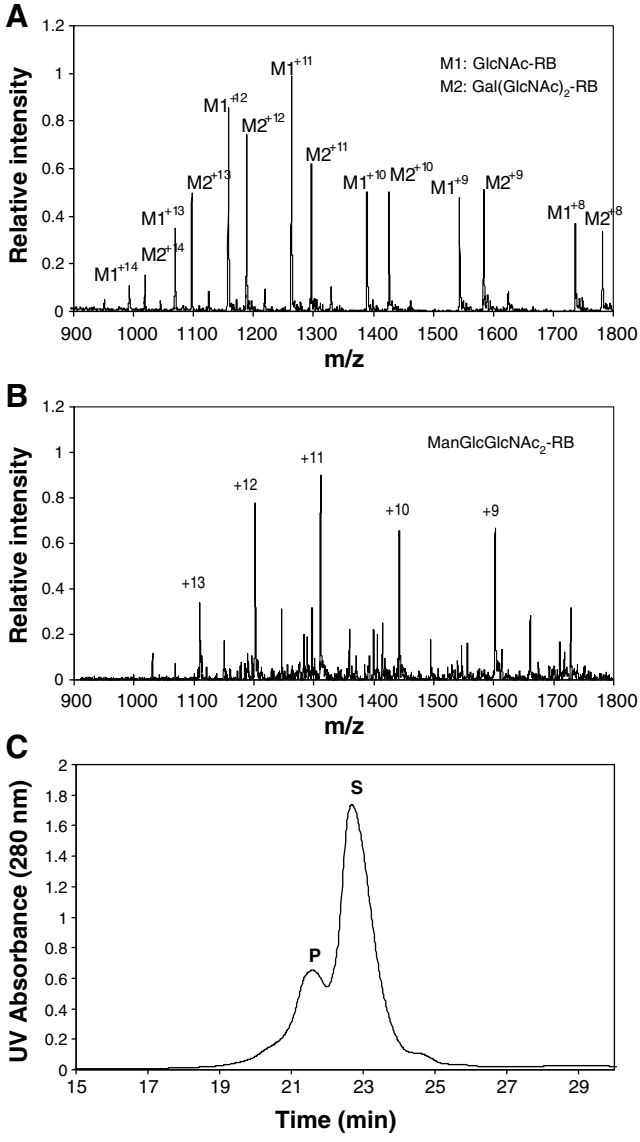


Figure 4. The ESI-MS spectra and HPLC profile of the synthetic neoglycoproteins. (A) The mixture of $Gal(GlcNAc)_2$-RB (**16**) and GlcNAc-RB; (B) the purified neoglycoprotein $ManGlc(GlcNAc)_2$-RB (**17**); (C) the HPLC profile of a typical transglycosylation reaction between oxazoline **13** and GlcNAc-RB (**15**). The starting material GlcNAc-RB was marked as S (t_R = 22.7 min) and the newly formed product Manα1,3Glcβ1,4GlcNAcβ1,4GlcNAc-RB (**17**) was marked as P (t_R = 21.4 min) (see the experimental part for the details of HPLC condition).

4. Experimental

4.1. Materials and methods

Chitinase from *Bacillus* sp. was purchased from Wako Chemical, Japan. LacNAc was purchased from Sigma. The GlcNAc-Ribonuclease B (**15**) was prepared as previously reported.[21] LacNAc-oxazoline (**3**) was synthesized according to the reported procedure[17]; the synthesis of ManGlcNAc-oxazoline (**9**) and Man_3GlcNAc-oxazoline (**11**) was described previously[18]; and ManGlcGlcNAc-oxazoline (**13**) was synthesized according to the reported procedure.[23] All other reagents were purchased from Sigma/Aldrich and used as received.

Analytical RP-HPLC was performed on a Waters Nova-Pak C18 column (3.9 × 150 mm) at 40 °C. The column was eluted with a linear gradient 0–60% MeCN containing 0.1% trifluoroacetic acid for 20 min at the flow rate of 1 ml/min, unless otherwise specified. The ESI-MS Spectra were measured on a micromass ZQ-4000 single quadruple mass spectrometer.

4.2. Purification of chitinases

The chitinases (20 mg) were dissolved into 20 mM Tris–HCl (pH 8.0) with 100 μl protease inhibitor cocktail (Sigma). The samples were then centrifuged at 10,000g at 4 °C for 10 min to get rid of insoluble materials. The supernatants were then loaded on a Superdex 200 gel filtration column and fractionated by an AKTA FPLC system (GE Healthcare). Fractions (200 μl each) were collected and assayed for chitinolytic activity. Individual fractions that have the highest chitinase activity were then collected and were examined on SDS–PAGE gel. Three major protein bands, which have the size of 100 kDa, 60 kDa and 40 kDa were identified and named as Chi100, Chi60 and Chi40, respectively. Fractions that contained the pure form of these individual proteins were then pooled and stored at −80 °C for further use.

4.3. Enzymatic assays

4.3.1. Measurement of hydrolytic activity

Chitinases (0.5 μg) were incubated with $(GlcNAc)_2$-MU (**1**) (0.1 mM) in an acetate buffer (50 μl, 50 mM, pH 6.0) at 37 °C. Aliquots (5 μl) were taken at a pre-defined time and added to 200 μl of a glycine buffer (150 mM, pH 10.5) in a Falcon 96-well microtiter plate to terminate the reaction and to enhance the fluorescence quantum yield. The fluorescence was then measured on a 96-well fluorescence plate reader (Wallac 1470 Victor multilabel counter, EG&G Wallac) (excitation wavelength, 355 nm; emission wavelength, 460 nm).

4.3.2. Measurement of transglycosylation activity

The transglycosylation activity of chitinase with GlcNAc-MU (**4**) was performed as follows: individual chitinase fractions (0.5 μg each) were incubated with LacNAc-oxazoline (**3**) (glycosyl donor, 0.4 mM) and GlcNAc-MU (**4**) (glycosyl acceptor, 1 mM) in a phosphate buffer (50 μl, 20 mM, pH 7.0). Aliquots (5 μl) were taken at pre-defined time, added to 200 μl of a glycine buffer (150 mM, pH 10.5) to terminate the reaction and to enhance the fluorescence quantum yield. The fluorescence was then measured as described above.

4.4. Synthesis of glyco-asparagine derivatives through the chitinase-catalyzed transglycosylation

A typical procedure for the synthesis of glyco-asparagine derivative (8) was shown here: a solution of GlcNAc-Asn-Fmoc (**7**) (2 mg) and LacNAc-oxazoline (**3**) (4 mg in total, added in portions at intervals) in a phosphate buffer (50 mM, pH 6.5, 200 μl) containing Chi100 (30 μg) was incubated at 30 °C. The reaction was monitored by HPLC. After 8 h, the reaction was terminated by 10% TFA and the product was purified by preparative HPLC on a Waters preparative column (Symmetry 300, 19 × 300 mm) to afford the glyco-asparagine derivative **8** (1.8 mg, 55%). ^{1}H NMR (d_6-DMSO + 5% D_2O, 500 MHz): δ 7.84 (d, 2H, J = 8.0 Hz, Fmoc-ArH), 7.66 (m, 2H, Fmoc-ArH), 7.37 (t, 2H, J = 7.5 Hz, Fmoc-ArH), 7.28 (t, 2H, J = 7.5 Hz, Fmoc-ArH), 4.81 (d, 1H, J = 7.5 Hz, H-1 of GlcNAc-1), 4.38 (d, 1H, J = 7.5 Hz, H-1 of GlcNAc-2), 4.22 (d, 1H, J = 8.0 Hz, H-1 of Gal), 4.17 (m, 2H, Fmoc-CH_2), 3.81 (m, 1H, Fmoc-9-H), 1.80 (s, 3H, GlcNAc-CH_3), 1.73 (s, 3H, GlcNAc-CH_3). ESI-MS of **12**: calculated, M = 922.33; found, 923.14 $(M+H)^+$.

Glycoasparagine derivative **14** was prepared in the similar way using Manα1,3Glcβ1,4GlcNAc-oxazoline (**13**) as the donor substrate (Yield, 27%). ^{1}H NMR (d_6-DMSO + 5% D_2O, 500 MHz): δ 7.82 (d, 2H, J = 8.0 Hz, Fmoc-ArH), 7.66 (m, 2H, Fmoc-ArH), 7.38 (t, 2H, J = 7.5 Hz, Fmoc-ArH), 7.30 (t, 2H, J = 7.5 Hz, Fmoc-ArH), 5.02 (s, 1H, H-1 of Man), 4.81 (d, 1H, J = 7.5 Hz, H-1 of GlcNAc-1), 4.38 (d, 1H, J = 7.5 Hz, H-1 of GlcNAc-2), 4.26 (d, 1H, J = 8.0 Hz, H-1 of Glc), 4.17 (m, 2H, Fmoc-CH_2), 1.80 (s, 3H, GlcNAc-CH_3), 1.74 (s, 3H, GlcNAc-CH_3). ESI-MS of **14**: calculated, M = 1084.39; found, 1085.25 $(M+H)^+$.

4.5. Synthesis of neoglycoproteins through the chitinase-catalyzed transglycosylation

A solution of LacNAc-oxazoline (**3**) (0.5 mg in total, added in portions) and GlcNAc-RB (**15**) (0.3 mg) in a phosphate buffer (50 mM, pH 6.5, 100 μl) containing Chi100 (1 μg) was incubated at 30 °C for 6 h. ESI-MS showed that the corresponding neoglycoprotein Gal-$GlcNAc_2$-RB (**16**) was formed (ca. 40%). But the product was co-eluted with the starting material GlcNAc-RB (**15**) under several HPLC conditions tested, making it difficult to obtain the neoglycoprotein in pure form. ESI-MS of **16**: calculated, M = 14251; found, 1782 $(M+8H)^{8+}$, 1585 $(M+9H)^{9+}$, 1426 $(M+10H)^{10+}$, 1297 $(M+11H)^{11+}$, 1188 $(M+12H)^{12+}$, 1097 $(M+13H)^{13+}$, 1019 $(M+14H)^{14+}$.

The transglycosylation between the Man-Glc-GlcNAc-oxazoline (**13**) and the GlcNAc-RB (**15**) in the presence of chitinase Chi100 was performed in the same way as described above for the synthesis of Gal-$(GlcNAc)_2$-RB (**17**). In this case the neoglycoprotein was eluted slightly earlier than the GlcNAc-RB (**15**) under an appropriate RP-HPLC condition. The analytical RP-HPLC was performed on a Waters Nova-Pak C18 column (3.9 × 150 mm) at 40 °C. The column was eluted with a linear gradient of 23–29% MeCN containing 0.1% trifluoroacetic acid for 30 min at the flow rate of 1 ml/min. The transglycosylation product was purified by RP-HPLC to give the neoglycoprotein Man-Glc-$(GlcNAc)_2$-RB (**17**) in 20% yield. ESI-MS of **17**: calculated, M = 14413; found, 1602 $(M+9H)^{9+}$, 1442 $(M+10H)^{10+}$, 1311 $(M+11H)^{11+}$, 1202 $(M+12H)^{12+}$, 1109 $(M+13H)^{13+}$, 1030 $(M+14H)^{14+}$.

Acknowledgements

The work was supported by the National Institutes of Health (R01 GM080374).

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