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A simplified procedure for gram-scale production of sialylglycopeptide (SGP) from egg yolks and subsequent semi-synthesis of $Man_3GlcNAc$ oxazoline

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ABSTRACT

Heterogeneity of glycan structures in native glycoconjugates always hampers precise studies on carbohydrate-involved biological functions. To construct homogeneous glycoconjugates from natural resource of homogeneous glycans is therefore a practical approach to solve this problem. We report here an optimized procedure for gram-scale production of sialylglycopeptide (SGP) containing a disialyl biantennary complex-type N-glycan from egg yolks. Our new procedure simplified the extraction process by treating the egg yolk powder with 40% acetone, avoiding massive emulsification, high-speed centrifugation, and sophisticated chromatography in reported methods. Subsequent semi-synthesis of the N-glycan core $Man_3GlcNAc$ oxazoline from SGP was accomplished for the first-time via glyco-trimming and successive oxazoline formation. This efficient semi-synthesis provides an alternative to the pure chemical approach that involves multi-step total synthesis and facilitates the application of *endo*-glycosidase-enabled chemoenzymatic synthesis of various homogeneous glycoconjugates.

1. Introduction

Glycosylation as a major post-translational modification of protein is involved in many important biological processes such as protein folding and quality control,[1–4] cell adhesion and migration,[5,6] viral entry and pathogenesis,[7,8] immune response and regulation,[9,10] etc. More than 50% of mammalian proteins and about 70% of clinical protein drugs are glycoproteins.[11] It has been tremendously demonstrated that glycan substructures may significantly affect functions of glycoproteins. However, heterogeneity of glycan structures in natural glycoproteins hampers the functional studies on precise structure–function relationship. As a solution to this problem, an efficient chemoenzymatic method for homogeneous glycoprotein synthesis has emerged, which takes advantage of the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidases,[11–16] a class of endoglycosidases that hydrolyze the glycosidic bond in the *N*,*N*′-diacetylchitobiose motif of N-glycans, in the presence of glycan donor substrates.

Yamamoto et al.[17–19] reported the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) using the egg-yolk sialylglycopeptide (SGP, Fig. 1)[20,21] as the donor substrate. The Endo-M enzyme cleaves the biantennary disialyl complex-type N-glycan from SGP and transfers it onto another GlcNAc-containing peptide/protein forming a new homogeneous glycopeptide/glycoprotein though in a relatively low yield. Thereafter, Shoda et al.[22] firstly discovered the synthetic glycan oxazoline, which is the transit-state form of *endo*-glycosidase-catalyzed N-glycan hydrolysis, could also serve as the transglycosylation donor substrate and dramatically improve the efficiency. Wang's and Fairbanks' groups synthesized a series of N-glycan oxazolines[23–32] including the core tetrasaccharide $Man_3GlcNAc$ oxazoline (Fig. 1) as the substrates of Endo-A from *Arthrobacter protophormiae*[33] that promoted excellent transglycosylation yields of resulting homogeneous glycopeptides and glycoproteins.

This chemoenzymatic transglycosylation approach presents a powerful tool for efficient synthesis of diverse glycoconjugates as summarized in Figure 1. Wang and his co-workers have made significant contributions to this method and successfully expanded its application in synthesis of important therapeutic glycoproteins,[34,35] glycopeptides[36–38], novel glyco-clusters,[39] glyco-natural products,[40] etc. (Fig. 1.) Recently, two papers have

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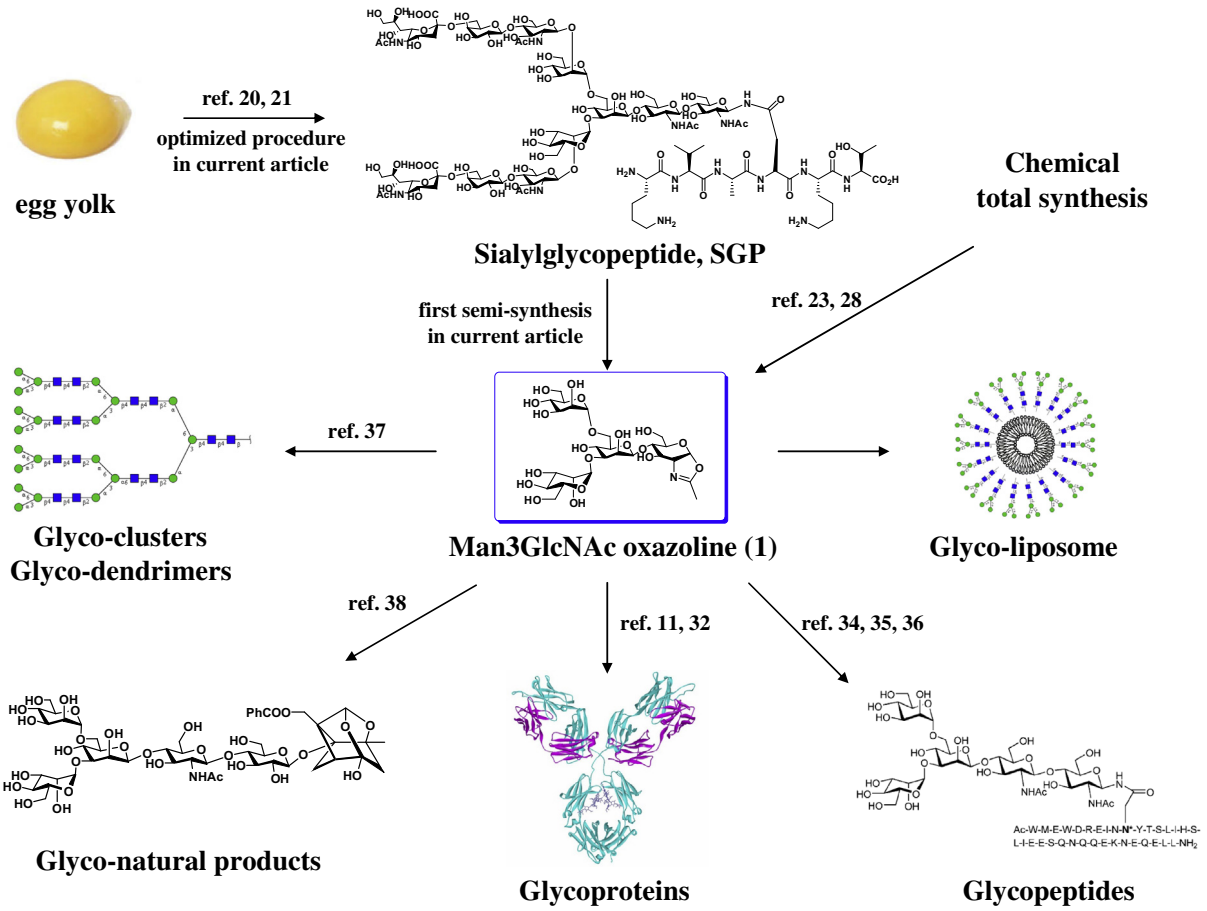


Figure 1. Preparation of N-glycan core Man3GlcNAc oxazoline and its application in chemoenzymatic synthesis of various glycoconjugates.

respectively reported the Fc-specific glycan remodeling of the monoclonal antibody drugs by Endo-S from *Streptococcus pyogenes* using $Man_3GlcNAc$ oxazoline as the homogeneous glycan donor.[11,34]

$Man_3GlcNAc$ oxazoline as the core N-glycan oxazoline has been employed in chemoenzymatic synthesis of various glycoconjugates (Fig. 1) and its chemical total synthesis has been reported by Wang's and Fairbanks' groups, respectively.[23,28] The total synthesis consists of over 30 synthetic steps with sophisticated process of regio-selective protection and deprotection, as well as stereo-selective glycosylation. The difficulty of long-step total synthesis in poor economic efficiency hinders the application of chemoenzymatic synthesis of glycoconjugates using the N-glycan oxazoline substrates. As an alternative option, semi-synthesis from natural homogeneous N-glycan resources was committed to prepare high-mannose type and biantennary complex-type N-glycan oxazolines in good yields.[41,42] Here, we report for the first time the expeditious semi-synthesis of core $Man_3GlcNAc$ oxazoline from the egg-yolk sialylglycopeptide (SGP) via enzymatic glyco-trimming and successive oxazoline formation reaction in a water solution.

SGP was firstly isolated by Seko et al. from egg yolks via phenol-extraction, centrifugal separation, ethylacetate wash, and comprehensive size-exclusion chromatography.[20] Zou et al. simplified the later-stage purification procedure using porous graphite carbon (PGC) extraction instead of reiterant gel-filtration operation.[21] However, the problem of emulsification in these methods caused a lot of trouble during all the processes before gel-filtration. Additionally, the requirement of long-time high-speed centrifugation for separation of phenol-extracting solution from precipitates limits the large-scale production. Sugawara et al. reported in a patent[43] using water to extract defatted egg yolks and treating the supernatant with alcohols or other organic solvents to precipitate SGP. But this method encounters the problems of emulsification, high-speed centrifugation, and complicated purification procedures as well. We developed a newly optimized procedure for gram-scale SGP preparation by reducing emulsification and averting centrifugation. This simplified method provides efficient scale-up of SGP extraction for potential industrial interest and facilitates expeditious semi-synthesis of N-glycan core $Man_3GlcNAc$ oxazoline for chemoenzymatic preparation of various glycoconjugates.

2. Results and discussion

2.1. Optimized procedure for gram-scale production of SGP

In Seko's approach, 9% phenol aqueous solution was employed to precipitate the unrelated proteins and extract the SGP from egg yolks. While, the tremendous emulsification occurred in the mixture probably is due to the blending of lipids, soluble proteins, organic solvent, and water. The extracts were highly emulsified so that separation of precipitates and supernatant was very difficult and incomplete even under high-speed (10,000*g*) centrifugation. A similar trouble also occurred when ethyl acetate was added to wash the supernatant after centrifugation. Thus, we sought to optimize the procedure to reduce or even avoid the emulsification for large-scale process.

Firstly, we lyophilized the egg yolks and washed the resulted yolk powder with diethyl ether to remove lipids that may lead to emulsification dramatically. Next, for SGP extraction, we chose acetone–water solution which generates much less emulsion than phenol–water or acetonitrile–water solutions. To search the right ratio of acetone–water solution for extraction, we used HPLC to scout the SGP content in yolk extracts from 80%, 70%, 60%, 40% acetone, and 9% phenol (Fig. 2).

There were no detectable SGP but other unidentified substances in yolk extracts by 80% or 70% acetone (Fig. 2a and b). When increasing the water portion in extracting solvent, SGP was pulled out from the yolk cake by 60% and 40% acetone as shown in Figure 2c and d (the SGP peak was marked with a star symbol). Compared with extract by 9% phenol (Fig. 2f), there are much less unrelated peaks in the HPLC profiles of 40% acetone and much less emulsification during the handling as well. We further investigated extracts by 50%, 30%, and 20% acetone and quantified the SGP contents in all extracts by HPLC (Table 1). The extraction rates of SGP in 40%, 30%, and 20% acetone indicated the best results attaining 1.02, 1.13, and 1.08 mg per gram egg yolk lyophilized powder, against 0.71 mg SGP per gram yolk powder by 9% phenol. In the end, we chose 40% acetone as the optimal extraction condition because of these reasons: (1) the SGP content in total extract weight by 40% acetone was about 5.8% which was the most efficient in all extraction conditions (Table 1); (2) SGP in extracts by 60% and 50% acetone were much less (Fig. 2 and Table 1); (3) emulsification gradually occurred in extraction procedure by 30% and 20% acetone but not by 40% acetone. To test the completion of SGP extraction by 40% acetone, we performed second-round extraction and determined the HPLC profile (Fig. 2e) indicating no more detectable SGP left in the precipitates after first extraction.

Table 1
SGP content determination

Extraction condition	SGP content in extracts (mg/1 g yolk powder)	Total weight of extracts (mg/1 g yolk powder)
70% Acetone	0	10.7
60% Acetone	0.38	13.2
50% Acetone	0.62	15.5
40% Acetone	1.02	17.6
30% Acetone	1.13	28.6
20% Acetone	1.08	37.9
9% Phenol	0.71	55.4

After extracted by 40% acetone, the residue yolk cake could be simply filtered through a layer of celite, averting emulsification trouble and high-speed centrifugal separation. Thereafter, we tried the purification of SGP via three different approaches, respectively: gel filtration with a Sephadex G-25 column, preparative HPLC isolation with a C18 column, and solid-phase extraction with an active carbon column. All these approaches could successfully isolate the pure SGP in similar yields. The procedure of active carbon extraction was most convenient with highest separation capability and less costly, while HPLC and gel filtration have limited sample loading and often required second-round purification especially

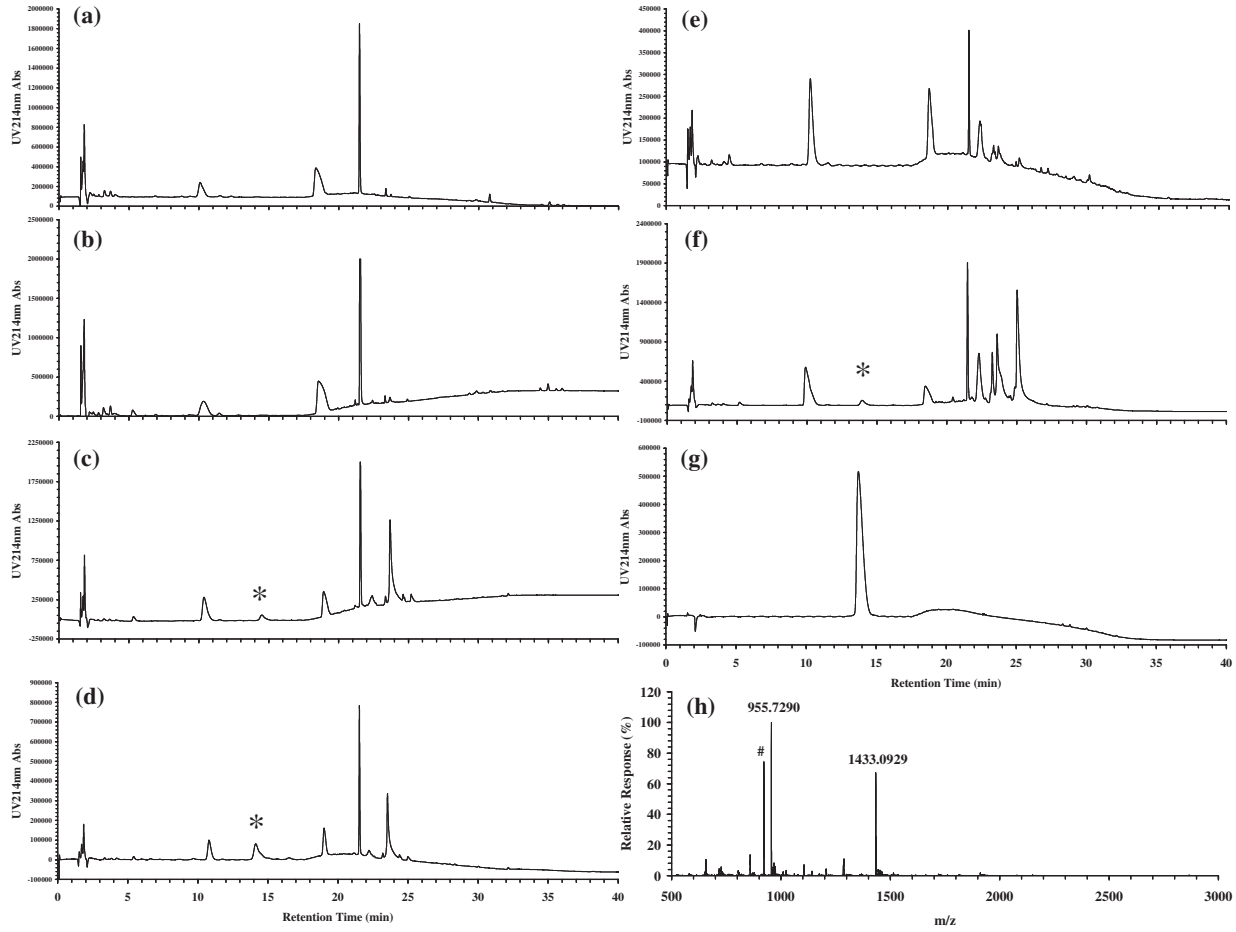


Figure 2. HPLC profiles of egg yolk extracts by different extracting solvents and ESI-HRMS of purified SGP. (a) extracts by 80% acetone; (b) 70% acetone; (c) 60% acetone; (d) 40% acetone; (e) second extracts by 40% acetone; (f) 9% phenol; (g) HPLC profile of purified SGP; (h) ESI-HRMS data of purified SGP. The SGP peak in HPLC profiles is marked with *; the HRMS reference is marked with # in the MS data.

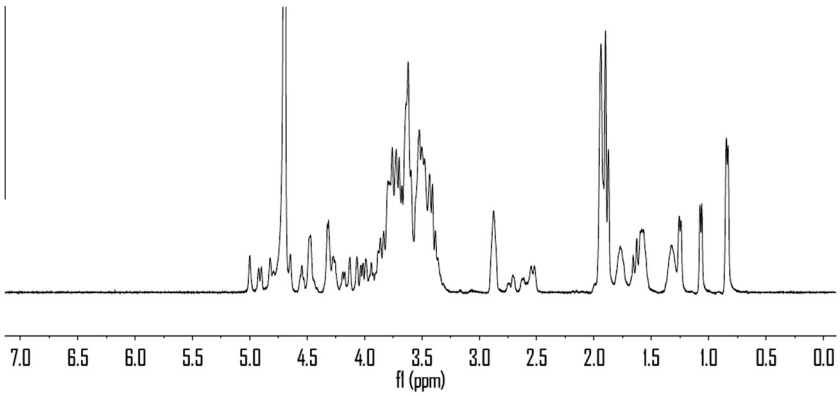
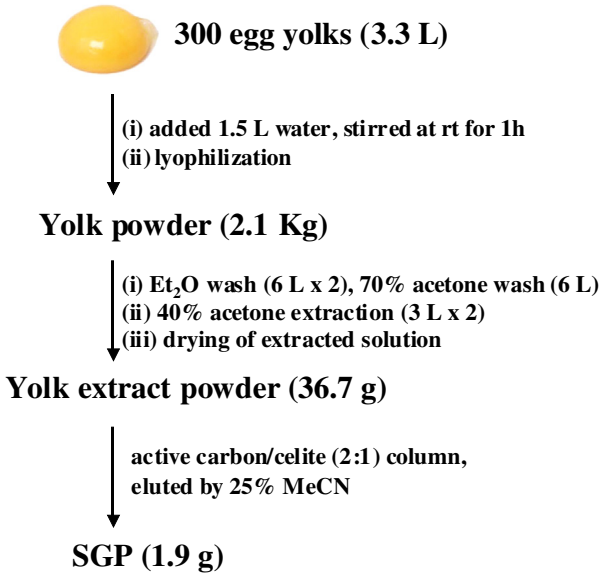


Figure 3. ^{1}H NMR spectrum of egg-yolk SGP.



Scheme 1. Optimal procedure for gram-scale SGP production.

for overloaded cases. The purified SGP was characterized by HPLC, HRMS, and NMR. The HPLC profile showed a single symmetry peak (Fig. 2g) suggesting a good purity. The ESI-HRMS spectrum (Fig. 2h) indicated *m*/*z* values of 1433.0929 and 955.7290 which are highly accordant with calculated SGP 2-charge mass 1433.0933 and 3-charge mass 955.7308. The NMR spectrum (Fig. 3) is also identical to the reference data.

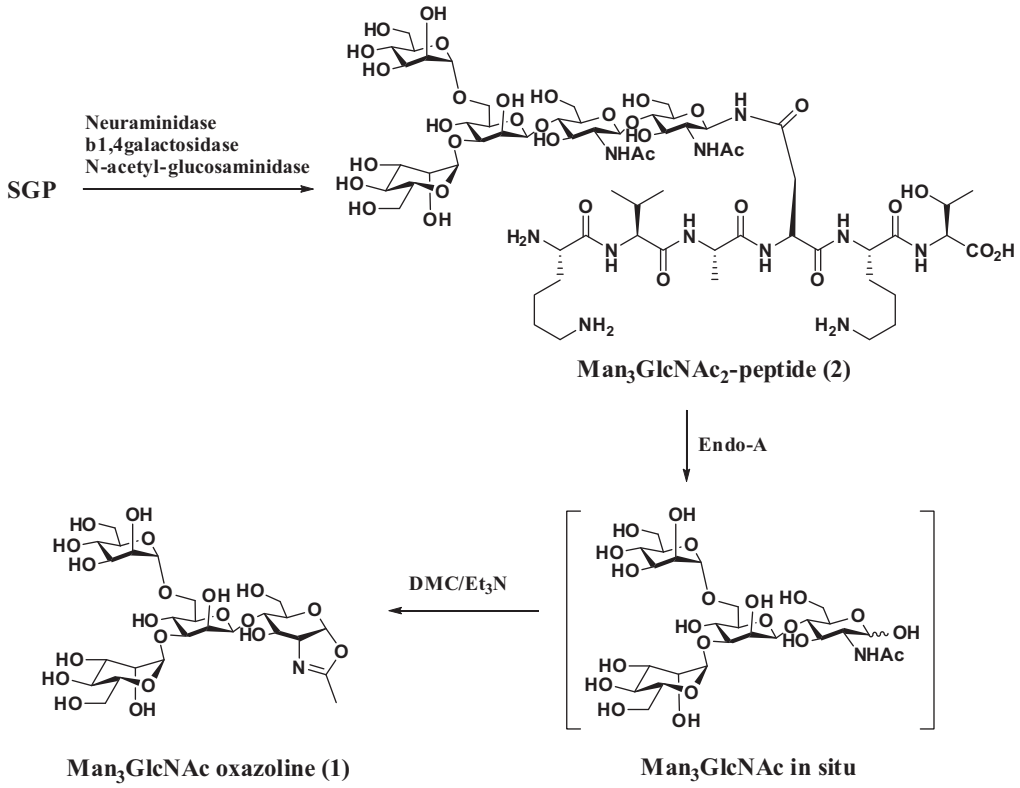
The optimal procedure for gram-scale SGP production is summarized in Scheme 1. Three hundred egg yolks were stirred with water and freeze dried to yolk powder. The powder was washed successively with ethyl ether and 70% acetone after thorough mixing. Thereafter, 40% acetone was added to the residue and mixed rigorously, then the solution was filtered through celite. The filtrate was concentrated and lyophilized. The resulted extract powder was dissolved in water and subject to solid-phase extraction with an active carbon/celite (2:1) column. The eluting solution by 25% MeCN gives the pure SGP in a yield of 0.9 g/kg yolk powder. In the references, the SGP yields were reported as 8 mg/yolk[20] or 680 mg/100 yolks,[21] however, it is too rough to compare the SGP content in each egg yolk because of the individual differences in egg batches. For example, the volume of 115 egg yolk was 1.9 L in the reference,[20] but only about 1.3 L in our case suggesting differences in size of eggs used. Thus we normalized the yield of SGP in per gram or kilogram egg yolk powder.

2.2. Efficient semi-synthesis of N-glycan core $Man_3GlcNAc$ oxazoline

With plenty amount of SGP in hand, we sought to develop a new semi-synthetic approach to prepare the N-glycan core $Man_3GlcNAc$ oxazoline which is widely applied in chemoenzymatic synthesis of various homogeneous glycoconjugates. Our strategy was to truncate the non-reducing end saccharide moieties by specific glycosidase enzymes to give the $Man_3GlcNAc_2$-peptide (**2**). Then, the core $Man_3GlcNAc$ glycan was cleaved from **2** by Endo-A and transformed to the oxazoline following Shoda's approach[44] using DMC (2-chloro-1,3-dimethyl-imidazolinium chloride) in presence of Et_3N (Scheme 2).

exo-Glycosidases were widely used to trim non-reducing end carbohydrate moieties of N-glycans for diverse glycan structures.[45–47] Firstly, we treated the SGP with neuraminidase to cleave the two sialic acids and monitored by HPLC and ESI-HRMS which clearly showed the complete removal of sialic acid (Fig. S1). Then β1,4galactosidase was added to trim exposed galactose moieties on the non-reducing end. After confirmation on the galactose digestion, *N*-acetyl-glucosaminidase was introduced for truncation on non-reducing end GlcNAc residues with β1,2-linkage to the mannoses, giving $Man_3GlcNAc_2$-peptide (**2**) after being isolated by preparative HPLC. All the processes of enzymatic glyco-hydrolysis were controlled step-by-step under HPLC and MS determination (Fig. S1). Since these three enzymes maintain good activity in the same phosphate buffer (pH 6.0), we performed the one-pot enzymatic hydrolysis by adding all three enzymes in the solution and successfully attained the desired product **2** in a good yield.

The N-glycan core $Man_3GlcNAc$ was then released from glycopeptide **2** catalyzed by glycosidase Endo-A in a pH 6.0 phosphate buffer. The high-resolution mass spectrum indicated *m*/*z* value of 730.2378 and 863.4834 in excellent agreement with theoretical mass of the release glycan [M+Na] 730.2382 and GlcNAc-peptide [M+H] 863.4838. After lyophilization, the obtained $Man_3GlcNAc$ in situ was directly transformed to its oxazoline form in a water solution following the reference. The reaction was performed in D_2O in the presence of DMC and Et_3N. ^{1}H NMR monitoring of the reaction showed H1 proton of GlcNAc as a doublet peak at 5.95 ppm with a coupling constant of 7.2 Hz that is the characteristic signal of GlcNAc anomeric proton in the oxazoline form. The integration of a full proton for this peak suggested the completion of oxazoline formation. These data implicated the efficient one-pot oxazoline formation in spite of the presence of buffer, peptide, and enzyme remained in the reaction. The oxazoline (**1**) was purified by solid-phase extraction via a PGC cartridge and the proton NMR spectrum as shown in Figure 4 is identical to the reference. This semi-synthesis provided an efficient approach for rapid



Scheme 2. Semi-synthesis of $Man_3GlcNAc$ oxazoline from SGP.

preparation of the core N-glycan oxazoline compared with long-step total synthesis.

2.3. Chemoenzymatic transglycosylation with Man3GlcNAc oxazoline

Using the $Man_3GlcNAc$ oxazoline prepared by our new method as the donor substrate, we repeated the reported experiment[25] on chemoenzymatic glycosylation of ribonuclease (RNase) B, a glycoprotein with catalytic activity for RNA degradation. N-Glycans of native RNase B is a mixture of $Man_{5-9}GlcNAc_2$ which could be rapidly cleaved by Endo-A giving GlcNAc-RNase. Endo-A could also expeditiously catalyze the transglycosylation of $Man_3GlcNAc$ oxazoline to GlcNAc-RNase forming a new homogeneous glycoform of RNase. We performed the one-pot glycan remodeling by mixing native $Man_{5-9}GlcNAc_2$-RNase, Endo-A, and $Man_3GlcNAc$ oxazoline in a pH7.0 phosphate buffer. After 1 h incubation at 37 °C, $Man_3GlcNAc_2$-RNase was given as expected (see Supporting information).

Endo-A has diverse substrate specificity in acceptor structures including GlcNAc-containing proteins, peptides, natural products, glycoclusters, etc. We synthesized an O-GlcNAc-amino acid to test the chemoenzymatic transglycosylation of $Man_3GlcNAc$ oxazoline to an O-GlcNAc moiety. The Fmoc-Ser(O-GlcNAc)-OH (**4**), prepared

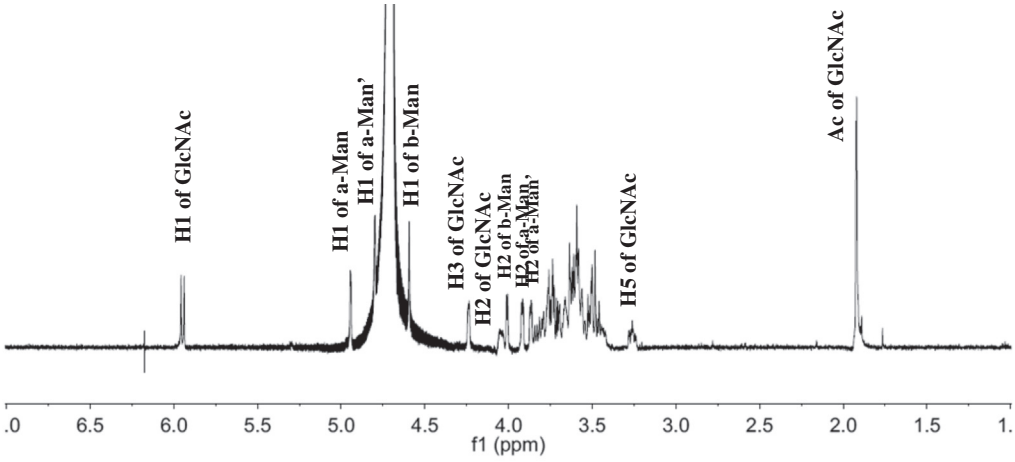


Figure 4. ^1H NMR spectrum of $Man_3GlcNAc$ oxazoline.

following the reported method (see Supporting information), was incubated with oxazoline (**1**) and Endo-A. The LC–MS data showed clearly the efficient glycosylation giving a new peak of Fmoc-Ser(O-$Man_3GlcNAc_2$)-OH confirmed by the HRMS data (see Supporting information). In biomedical proteomic studies, the attachment of $Man_3GlcNAc$ moiety could be useful for enrichment of O-GlcNAc glycopeptides by mannose-binding lectins. A related research is undergoing.

3. Conclusion

An optimized procedure for the gram-scale production of SGP from egg yolks was successfully achieved that expedites the subsequent semi-synthesis of N-glycan core $Man_3GlcNAc$ oxazoline compared with long-step total synthesis. This approach provides the rapid preparation of homogeneous N-glycan substrates from natural resource and facilitates the following chemoenzymatic synthesis of homogeneous glycoprotein and other glycoconjugates.

4. Experimental section

4.1. General

Chemical reagents and solvents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China) and used without further purification. HPLC grade acetonitrile was purchased from Meryer (Shanghai, China). Neuraminidase and β-1,4-galactosidase were purchased from New England Biolabs (Ipswich, MA). β-*N*-Acetylglucosaminidase and ribonuclease B was purchased from Sigma–aldrich (Shanghai, China). *endo*-β-*N*-Acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was purchased from Accendatech Co. (Tianjin, China). The unit definitions of enzyme activities were following the description from manufacturers. Porous graphite carbon (PGC) cartridges (Hypercarb) were purchased from Thermo Fisher Scientific (Beijing, China). Fresh egg yolks were obtained from hen's eggs purchased from local market. TLC staining for carbohydrate samples was performed by dipping the plates into 10% H_2SO_4 in methanol and drying with a heat gun. Nuclear magnetic resonance (NMR) spectra were measured on a Varian-MERCURY Plus-400 instrument. The chemical shifts were assigned in ppm and the coupling constants in Hz. ESI-MS spectra were measured on an Agilent 6110 single quadrupole spectrometer. ESI-HRMS spectra were measured on an Agilent 6230 LC–TOF MS spectrometer. The deconvolution MS data were generated by MagTran 1.02.[48]

4.2. High-performance liquid chromatography (HPLC)

Analytical RP-HPLC was performed on two instruments with different columns. Method A was performed on an Angilent 1260 UHPLC system with a C18 column (1.8 μm, 3.0 × 100 mm) at 32 °C. The column was eluted with an isocratic 2% acetonitrile containing 0.1% TFA in first 5 min then a linear gradient of 2–5% acetonitrile containing 0.1% TFA in additional 10 min at a flow rate of 1 mL/min. Method B was performed on a Beijing ChuangXinTongHeng LC3000 (analytic) instrument with a C18 column (5 μm, 4.6 × 150 mm) at 40 °C. The column was eluted with a linear gradient of 2–5% acetonitrile containing 0.1% TFA in first 15 min then 5–90% acetonitrile in additional 15 min at a flow rate of 1 mL/min. Preparative HPLC was performed on a Beijing ChuangXinTongHeng LC3000 (preparative) instrument with a preparative C18 column (10 μm, 20 × 300 mm). The column was eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA at a flow rate of 10 mL/min.

4.3. Preparation of egg yolk powder

Fresh 300 egg yolks were separated from egg white carefully. Water (1.5 L) was added and the mixture was stirred at rt for 1 h. The residue was subject to a freeze drier for lyophilization until complete dryness, giving the yellow egg yolk powder 2.1 kg.

4.4. Extraction condition optimization on SGP isolation from egg yolk powder

To develop an optimal isolation method for SGP production, we tried various extraction conditions and quantified the SGP contents in all extracts by HPLC.

4.4.1. Extraction by 9% phenol

Yolk powder (100 g) was mixed with 9% aqueous phenol solution (300 mL) and the mixture was vigorously stirred for 2 h with an ice bath. The residue in massive emulsion was centrifuged at 10,000*g* for 30 min. The less emulsive supernatant was separated from the precipitates and subject to second-round centrifugation. The final supernatant was washed with ethyl acetate (300 mL × 3). The aqueous layer was lyophilized to give a pale yellow powder (5.54 g). The resulted yolk extract powder was subject to analytic HPLC for SGP content determination.

4.4.2. Extraction by various acetone–water solutions

Yolk powder (100 g) was mixed with ethyl ether (300 mL) and stirred vigorously at rt for 30 min. The ether layer was removed by filtration and the solid was mixed with additional ethyl ether (300 mL) and the processes of wash and filtration were repeated. The pellet residue was respectively subject to 80%, 70%, 60%, 50%, 40%, 30%, and 20% acetone (150 mL twice) extraction. The extract solution was filtered and lyophilized to give the yolk extract powders under different conditions. The SGP content of each extract powder was measured by HPLC and the results are summarized in Figure 2 and Table 1.

4.5. Purification of SGP from yolk extract powder from 40% acetone

4.5.1. Gel-filtration with a Sephadex G-25 column

Yolk extract powder from 40% acetone (1 g) was dissolved in 5 mL water and subject to a Sephadex G-25 column, eluted by 0.1 M AcOH. The fractions containing pure SGP were combined and lyophilized. The fractions containing SGP and other impurities were subject to second-round gel-filtration purification. All pure SGP was combined and lyophilized to give a white powder (48 mg). The HPLC, NMR, and MS data indicated the purity of SGP was more than 95%.

4.5.2. Preparative HPLC with a C18 column

Yolk extract powder from 40% acetone (1 g) was dissolved in 5 mL water and subject to the preparative HPLC system, eluted by an isocratic water solution containing 0.1% TFA for 10 min then a linear gradient of 0–10% acetonitrile containing 0.1% TFA for additional 20 min. The fractions containing pure SGP were combined and lyophilized. The fractions containing SGP and other impurities were subject to second-round HPLC purification. All pure SGP was combined and lyophilized to give a white powder (51 mg). The HPLC, NMR, and MS data indicated the purity of SGP was more than 95%.

4.5.3. Solid-phase extraction with an active carbon/celite column

Yolk extract powder from 40% acetone (1 g) was dissolved in 5 mL water and subject to an active carbon/celite (2:1) column

(2 × 10 cm). The column was washed by water containing 0.1% TFA (50 mL), 5% acetonitrile containing 0.1% TFA (50 mL), and 10% MeCN containing 0.1% TFA (50 mL). Then, it was eluted by 25% acetonitrile containing 0.1% TFA (50 mL). The elution fractions were combined and lyophilized to give SGP as a white powder (52 mg). The HPLC, NMR, and MS data indicated the purity of SGP was more than 95%.

4.6. Optimized procedure for gram-scale SGP production

Yolk powder (2.1 kg) was washed thoroughly with ethyl ether (6 L × 2) and 70% acetone (6 L). The residue was extracted by 40% acetone (3 L × 2) and filtered. The filtrate was concentrated and subject to an active carbon/celite (2:1) column purification, giving SGP as a white powder (1.9 g, yield 0.9 g/kg yolk powder). Analytic HPLC: t_R = 13.7 min (Method A); t_R = 11.3 min (Method B); ESI-HRMS: calcd for $C_{112}H_{189}N_{15}O_{70}$ $[M+2H]^{2+}$ 1433.0923, found 1433.0929, $[M+3H]^{3+}$ 955.7308, found 955.7290; ^1H NMR (D_2O,400 MHz): δ 4.96 (s, 1H, H1 of Man-4), 4.86 (d, J = 10.0 Hz, 1H, H1 of GlcNAc-1), 4.77 (s, 1H, H1 of Man-4′), 4.60 (s, 1H, H1 of Man-3), 4.50 (m, 1H), 4.44 (m, 3H, H1 of GlcNAc-2, 5, and 5′), 4.27 (m, 2H, H1 of Gal-6 and 6′), 2.83 (m, 4H, Lys-CH2), 2.68 (dd, J = 5.2, 16.0 Hz, 1H, Asn-betaH), 2.54 (dd, J = 7.2, 16.0 Hz, 1H, Asn-betaH), 2.49 (m, 2H, H3eq of NeuAc), 1.90–1.83 (m, 18H, Ac ×6), 1.73 (m, 4H, Lys-CH2), 1.62–1.51 (m, 6H, Lys-CH2, H3ex of NeuAc), 1.27 (m, 4H, Lys-CH2), 1.20 (d, J = 7.6 Hz, 3H, Ala-CH3), 1.01 (d, J = 6.4 Hz, 3H, Thr-CH3), 0.78 (d, J = 6.0 Hz, 6H, Val-CH3).

4.7. Synthesis of $Man_3GlcNAc_2$-peptide (2) by enzymatic digestion

SGP (500 mg, 0.17 mmol) in a phosphate buffer (50 mM, pH 6.0, 3.5 mL) was incubated with neuraminidase (1500 U) at 32 °C until LC–MS showed the desialylation was complete giving the asialoglycopeptide (ESI-HMRS: calcd for $C_{90}H_{155}N_{13}O_{54}$ $[M+2H]^{2+}$ 1141.9970, found 1141.9993; $[M+3H]^{3+}$ 761.6672, found 761.6669). To the reaction solution was added β-1,4-galactosidase (800 U) and the mixture was incubated at 32 °C until LC–MS showed the degalactosylation was complete giving the $GlcNAc_2Man_3GlcNAc_2$-peptide (ESI-HMRS: calcd for $C_{78}H_{135}N_{13}O_{44}$ $[M+2H]^{2+}$ 979.9441, found 979.9430; $[M+3H]^{3+}$ 653.6320, found 653.6310). The pH of the reaction was adjusted to 5.0 and β-*N*-acetylglucosaminidase (5 U) was added. The mixture was incubated at 25 °C until LC–MS showed the GlcNAc removal was complete. The residue was subject to preparative HPLC purification to give the $Man_3GlcNAc_2$-peptide (**2**) as a white powder (195 mg, 74%). ESI-HMRS: calcd for $C_{62}H_{109}N_{11}O_{34}$ $[M+H]^+$ 1552.7217, found 1552.7210; $[M+2H]^{2+}$ 776.8648, found 776.8639.

4.8. Synthesis of $Man_3GlcNAc$ oxazoline (1)

$Man_3GlcNAc_2$-peptide (195 mg, 0.125 mmol) in a phosphate buffer (50 mM, pH 6.0, 2 mL) was incubated with Endo-A (10 U) at 37 °C until LC–MS showed the complete cleavage, releasing $Man_3GlcNAc$ (ESI-HMRS: calcd for $C_{26}H_{45}NO_{21}$ $[M+H]^+$ 708.2562, found 708.2555, $[M+Na]^+$ 730.2382, found 730.2378) and GlcNAc-peptide (ESI-HMRS: calcd for $C_{36}H_{66}N_{10}O_{14}$ $[M+H]^+$ 863.4838, found 863.4834). The residue was treated with DMC (350 mg, 2.07 mmol) and Et_3N (0.85 mL, 6.1 mmol) and incubated at 0 °C for 1 h. The reaction solution was diluted by 1 mM aqueous NaOH solution and subject to solid-phase extraction with PGC cartridge (Hypercarb). After being washed with 5% and 10% MeCN containing 1 mM NaOH, the cartridge was eluted with 25% MeCN containing 1 mM NaOH to give the product $Man_3GlcNAc$ oxazoline as a white solid (82 mg, 95%). ^1H NMR (D_2O, 400 MHz): δ 5.95 (d, J = 7.2 Hz, 1H, H1 of GlcNAc), 4.94 (s, 1H, H1 of a-Man), 4.80 (s, 1H, H1 of a-Man′), 4.59 (s, 1H, H1 of b-Man), 4.23 (m, 1H, H3 of GlcNAc), 4.05 (m, 1H, H2 of GlcNAc), 4.01 (d, J = 2.8 Hz, 1H, H2 of b-Man), 3.91 (m, 1H, H2 of a-Man), 3.86 (m, 1H, H2 of a-Man′), 3.25 (m, 1H, H5 of GlcNAc), 1.92 (d, J = 1.6 Hz, 3H, Ac).

4.9. Chemoenzymatic synthesis of $Man_3GlcNAc_2$-RNase by one-pot glycan remodeling

Chemoenzymatic synthesis of $Man_3GlcNAc_2$-RNase was performed following the reported approach summarized below. Native RNase B (1 mg, 67 nmol) and $Man_3GlcNAc$ oxazoline (400 μg, 580 nmol) in a phosphate buffer (100 mM, pH 7.0, 15 μL) were incubated at 37 °C with Endo-A (0.1 U) for 1 h. LC–MS data indicated the transglycosylation product $Man_3GlcNAc_2$-RNase was obtained in about 80% yield. ESI-MS: calcd M = 14576.25, found $[M+7H]^{7+}$ 2083.0709, $[M+8H]^{8+}$ 1822.6968, $[M+9H]^{9+}$ 1620.7407, $[M+10H]^{10+}$ 1458.3588, $[M+11H]^{11+}$ 1325.9682, $[M+12H]^{12+}$ 1215.3837, $[M+13H]^{13+}$ 1122.2093; deconvolution MS 14575.73.

4.10. Chemoenzymatic synthesis of Fmoc-Ser(O-$Man_3GlcNAc_2$)-OH

Fmoc-Ser(O-GlcNAc)-OH (prepared following reported method as described in Supporting information, 26.5 μg, 50 nmol), $Man_3GlcNAc$ oxazoline (207 μg, 300 nmol) in a phosphate buffer (100 mM, pH 7.0, 28 μL) were incubated at 37 °C with Endo-A (0.2 U) for 1 h. LC–MS data indicated the transglycosylation product Fmoc-Ser(O-$Man_3GlcNAc_2$)-OH was obtained in about 85% yield. ESI-MS: calcd $C_{52}H_{73}N_3O_{30}$, $[M+H]^+$ 1220.4357, found 1220.4353.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.07.013.

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