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Chemoenzymatic Synthesis of High-Mannose Type HIV-1 Gp120 Glycopeptides

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Abstract—A chemoenzymatic approach to the synthesis of glycoforms of HIV-1 gp120 glycopeptides is described. Thus, the high-mannose type glycopeptides [gp120 (336–342)] containing Man_9, Man_6 and Man_5 moieties, respectively, were synthesized in satisfactory yields via transglycosylation to the acetylglucosaminyl peptide, using the recombinant *Arthrobacter* Endo-β-*N*-acetyl-glucosaminidase (Endo-A) as the key enzyme.

The oligosaccharide component of glycoproteins plays an important role in many biological processes such as cell adhesion, cell differentiation, host-pathogen interactions, and immune responses.[1–3] Glycosylation of proteins also affects protein's folding, stability, antigenicity and immunogenicity.[4,5] The human immunodeficiency virus type 1 (HIV-1) has two envelope glycoproteins that form trimeric complexes on the viral surface. The outer envelope glycoprotein gp120 is heavily glycosylated. It has 24 conserved *N*-glycosylation sites, where 13 are complex-type *N*-glycans, and 11 are high-mannose type and/or hybrid type *N*-glycans.[6–8] Recent studies have suggested that, in addition to their roles in immune evasion of HIV/AIDS,[9] the *N*-glycans on HIV also play an active role in molecular recognition in HIV pathogenesis. For example, the high-mannose type oligosaccharides on gp120 was found to serve as the ligands of DC-SIGN, a newly discovered dendritic cell-specific lectin that mediates the capture and transmission of HIV-1 from the mucosal sites of infection to T-cells at secondary lymphenoid tissues, implicating an important role of the oligosaccharides in HIV-1 attachment, infection, and transmission.[10–12] Cyanovirin-N, a potent HIV-inactivating protein, exhibits its strong HIV-inhibitory activities through tight and specific binding to the high-mannose type oligosaccharides (Man_9 and Man_8) on gp120.[13–16] On the other hand, a cluster of two or more high-mannose oligosaccharides and/or glycopeptide moieties on gp120 was characterized as the epitope of the HIV-1 broadly neutralizing antibody 2G12.[17–19]

To further understand the function of these oligosaccharides in HIV pathogenesis, it is urgent to have an easy access to homogeneous gp120 glycopeptides as model compounds, particularly the glycopeptides of high-mannose type that are directly involved in HIV-1 pathogenesis. It was reported that glycosylation of gp120 is diverse and highly heterogeneous.[6,7] In addition to various complex type *N*-glycans, the high-mannose type *N*-glycans on gp120 range from Man_5 to Man_9, with Man_8 and Man_9 as the major glycoforms.[6,20] As the first step to determine whether the distinct glycoforms modulate the biological recognition, antigenicity, and immunogenicity of gp120, we describe in this paper a facile chemoenzymatic synthesis of HIV-1 glycopeptides containing Man_9, Man_6, and Man_5 *N*-glycans, respectively, using endo-β-*N*-acetyl-glucosaminidase from *Arthrobacter* (Endo-A) as the key enzyme.

Despite tremendous progress in recent years in the art of glycopeptide synthesis, the making of homogeneous glycopeptides containing large native oligosaccharide chains is still a difficult task.[5,20–23] Endo-β-*N*-acetyl-glucosaminidase from *Arthrobacter* (Endo-A) is a hydrolytic enzyme that releases high-mannose *N*-glycans from glycoproteins by cleaving the β-glycosidic

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linkage between the two GlcNAc residues in the *N*-glycans. But Endo-A also shows a significant trans-glycosylation activity.[24,25] That is, the enzyme can transfer an oligosaccharide moiety to a GlcNAc moiety (or other suitable acceptors) under certain conditions, forming a new β-1,4-glycosidic bond in the product.[26] Previously, we and others have described some applications of endo-enzyme catalyzed trans-glycosylation for synthesizing natural and non-natural glycopeptides.[27–32] The clear advantage of this chemo-enzymatic approach is that it allows the adding of a large oligosaccharide moiety to a GlcNAc-containing peptide in a single step. We have chosen the HIV-1 gp120 glycopeptide from the sequence 336–342 of gp120 as our first target. Glycopeptide gp120 (336–342) contains a high-mannose type oligosaccharide chain at the N339 glycosylation site and may constitute part of the epitope of the broadly HIV-1 neutralizing antibody 2G12, because mutation of this N-linked glycosylation site abolished the binding of gp120 to the neutralizing antibody.[17]

The *N*-acetylglucosaminyl peptide (336–342) **2** was prepared by solid-phase peptide synthesis using standard Fmoc-amino acids as the building blocks except for the Asn residue that was replaced with Fmoc-Asn(Ac_3GlcNAc)-OH as the building block in the synthesis. Cleavage of the peptide from the resin with simultaneous deprotection gave the corresponding *O*-acetylated *N*-acetylglucosaminyl peptide, which was purified by HPLC [ESI-MS of the derivative: 1174.82 $(M+H)^+$ and 588.15 $(M+2H)^{2+}$]. De-*O*-acetylation of the *O*-acetylated peptide by treatment with 5% hydrazine[33] and subsequent preparative HPLC afforded the *N*-acetylglucosaminyl peptide (336-342) **2** [ESI-MS: 1048.78 $(M+H)^+$ and 525.08 $(M+2H)^{2+}$]. On the other hand, the high-mannose oligosaccharide donor, Man_9GlcNA-c_2Asn (**1**), was prepared via thorough pronase digestion of soybean agglutinin that was isolated from soybean flour following the published method.[34,35] Transglycosylation reaction[36] between $Man_9GlcNAc_2Asn$ (**1**) and the *N*-acetylglucosaminyl peptide **2** was performed using the immobilized Endo-A[37] at 37 °C in an acetate buffer containing 25% acetone (Scheme 1). The reaction was monitored by HPLC (Fig. 1). When the reaction proceeded, a single new peak appeared preceding the peptide **2**, which was the transglycosylation product **3** (Fig. 1). After the size of the new peak reached the maximum (50 min), the reaction was stopped by heating and subsequent filtration. The product was purified by preparative HPLC and excess peptide **2** was readily recovered. The glycopeptide **3**[36] was identified by ESI-MS, which showed typical peaks at 2711.02 for $(M+H)^+$ and 1356.52 for $(M+2H)^{2+}$. The yield of **3** was 28% based on the glycosyl donor **1** and 93% based on the recovery of the acceptor **2**.

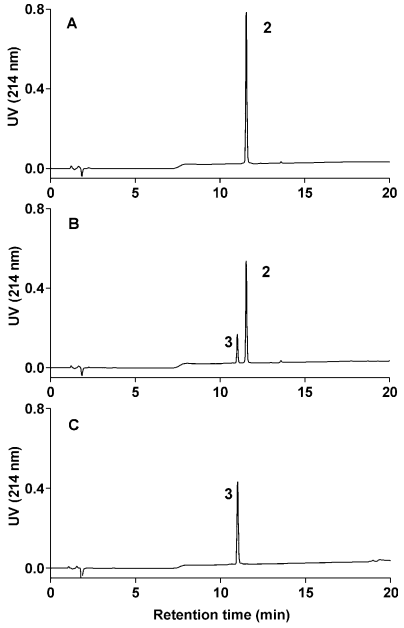
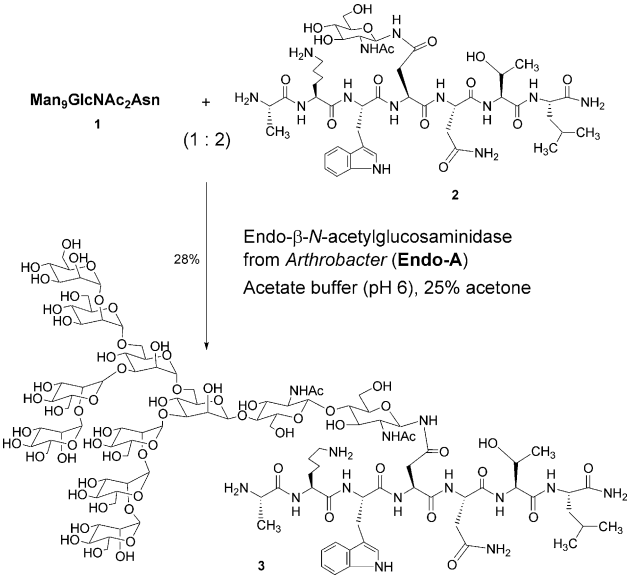


Figure 1. HPLC profiles of the transglycosylation reaction: (A) peptide **2**; (B) reaction mixture at 50 min; and (C) purified glycopeptide **3**. HPLC conditions: column, Waters Nova-Pak C18 column (3.9×150 mm); linear gradient, 0–90% MeCN containing 0.1% TFA in 25 min; flow rate: 1 mL/min.

To prepare additional glycoforms of high-mannose type glycopeptides found on HIV-1 gp120, the glycosyl donors $Man_6GlcNAc_2Asn$ **4** and $Man_5GlcNAc_2Asn$ **5** were prepared through pronase digestion of chicken ovalbumin with subsequent chromatographic purification of the digestive products **4** and **5**, according to the reported procedure.[38] Endo-A catalyzed transglycosylation between the glycosyl acceptor *N*-acetylglucosaminyl peptide **2** and the donor **4** or **5** was performed as for the preparation of glycopeptide **3**, giving the corresponding glycopeptides **6** and **7** in 25 and 27% yields, respectively (Fig. 2). Again the transglycosylation products were characterized by ESI-MS. Glycopeptide **6** showed typical peaks at 2224.80 $[(M+H)^+]$ and 1112.96 $[(M+2H)^{2+}]$, and the glycopeptide **7** showed peaks at 2062.80 $[(M+H)^+]$ and 1031.96 $[(M+2H)^{2+}]$ in their ESI-MS, respectively, which are in agreement with their structures.

We also compared the relative reactivity of the glycosyl donors $Man_9GlcNAc_2Asn$ **1,** $Man_6GlcNAc_2Asn$ **4,** and



Scheme 1. Synthesis of Man_9-glycopeptide **3**.

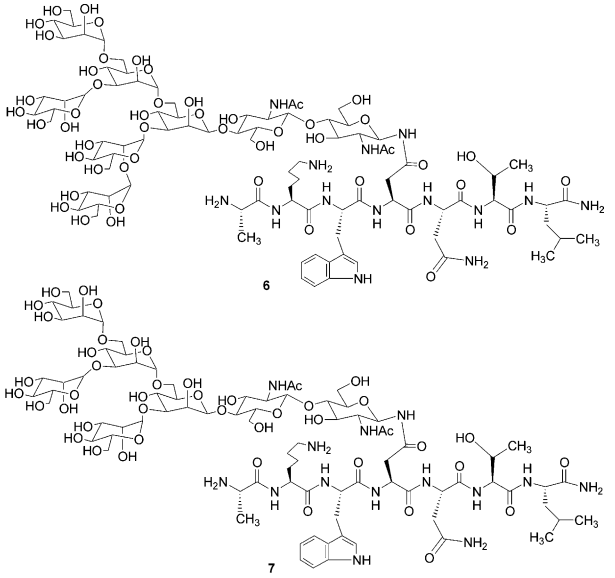


Figure 2. Structures of the synthetic Man_6- and Man_5-glycopeptides **6** and **7**, respectively.

$Man_5GlcNAc_2Asn$ **5** toward the acceptor **2** in the Endo-A catalyzed transglycosylation reaction. Equal molar amounts of **1**, **4**, and **5** were mixed with 10 molar excess of acceptor **2** in an acetate buffer (pH 6.0). The mixture was incubated with Endo-A and the reaction was monitored by HPLC. Interestingly, no apparent difference among the three glycosyl donors were found in the enzymatic reaction, and the corresponding transglycosylation products **3**, **6**, and **7** were generated at roughly the same reaction rate under the reaction conditions (data not shown). The results suggest that the enzymatic approach is equally efficient for the synthesis of an array of high-mannose type glycopeptides with varied size of the oligosaccharide moieties.

In summary, a facile synthesis of homogeneous high-mannose type gp120 glycopeptides of different size was described. The apparent advantage of the Endo-enzyme catalyzed method is the single-step transfer of an oligosaccharide moiety to an acceptor, which is in contrast to the common glycosyltransferase-catalyzed synthesis that adds only one monosaccharide moiety at a time. The gp120 glycopeptides **3**, **6**, and **7** synthesized are part of the antigenic structure of the HIV-1 broadly neutralizing antibody 2G12. Application of the chemoenzymatic approach to the synthesis of the complete 2G12-antigenic structure that contains more than one high-mannose oligosaccharide chains, as well as the 2G12-binding studies with the synthetic antigens are in progress.

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36. Experimental procedure for the synthesis of glycopeptide **3**: A mixture of the $Man_9GlcNAc_2Asn$ **1** (5 mg, 2.5 μmol) and Peptide **2** (5.3 mg, 5.0 μmol) in 50 mM ammonium acetate buffer (pH 6.0, 100 μL) containing 25% acetone was incubated at 37 °C with 25 milli-units of the recombinant enzyme GST-Endo-A that was immobilized on glutathione-Sepharose 4B beads. The reaction was monitored by analytical HPLC on a Waters Nova-Pak C18 column (3.9×150 mm) at 40 °C with a linear gradient (0–90% MeCN containing 0.1% TFA in 25 min, flow rate 1 mL/min). After 50 min, the reaction was stopped by heating in a boiling water bath for 3 min and filtered. The transglycosylation product was purified by preparative HPLC on a Waters preparative column (Symmetry 300, 19×300 mm) to afford the glycopeptide **3** (1.90 mg). In addition, 4.2 mg of peptide **2** was recovered from HPLC. ESI-MS of **3**: 2765 $(M+MeOH+Na)^+$, 2711.02 $(M+H)^+$, 2549.50 $(M-Man+H)^+$, 2386.50 $(M-2Man+H)^+$, 2224.52 $(M-3Man+H)^+$, 2062.50 $(M\text{-}4Man+H)^+$, and 1356.52 $(M+2H)^{2+}$
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