J. Microbiol. Biotechnol. (2003), **13**(2), 191–195

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vitro Glycosylation of Peptide (RKDVY) and RNase A by PNGase F

PARK, SU JIN, JI YOUN LEE, AND TAI HYUN PARK*

School of Chemical Engineering and Institute of Chemical Processes, Seoul National University, Kwanak-gu, Shilim-dong, San 56-1, Seoul 151-744, Korea

Received: July 16, 2002
Accepted: November 28, 2002

Abstract The *in vitro* glycosylation of pentapeptide (Arg-Lys-Asp-Val-Tyr; RKDVY) and RNase A was carried out using PNGase F (peptide-*N*-glycosidase F), and the results were analyzed using MALDI-TOF-MS. Aminated *N,N*-diacetyl chitobiose was used as the sugar in the glycosylation reaction, and the amination yield of *N,N'*-diacetyl chitobiose was about 60%. To reduce the water activity and shift the reaction equilibrium to a reverse reaction, 1,4-dioxane or ethylene glycol was used as the organic solvent in the enzymatic glycosylation. A certain extent of nonenzymatic glycosylation, known as the Maillard reaction, was also observed, which occurs on an arginine or lysine residue when the length of the sugar residue is one or two. However, the extent of glycosylation was much higher in the enzymatic reaction, indicating that PNGase F can be effectively used to produce glycopeptides and glycoproteins *in vitro*.

Key words: *In vitro* glycosylation, PNGase F (peptide-*N*-glycosidase F), pentapeptide (RKDVY), RNase A

Glycosylation is one of the most important post- and co-translational modifications which occur in eukaryotic cells. When a protein is expressed in eukaryotic cells and contains a sequence of asparagine-any amino acid-serine or asparagine-any amino acid-serine, it is likely to contain a sugar attachment at the amino side chain of asparagine (*N*-glycosylation). An attachment of sugar also occurs less commonly at a serine or threonine residue (*O*-glycosylation). Thus, glycosylation would appear to play a key role in maintaining a protein's overall structure, as well as having a direct effect on its activity [11]. Furthermore, glycosylation can increase the protein solubility, reduce the immune response, and protect the protein from an attack of protease, due to the features of the sugar chains, such as hydrophilicity and bulkiness [6].

*Corresponding author
Phone: 82-2-880-8020; Fax: 82-2-875-9348;
E-mail: thpark@plaza.snu.ac.kr

Glycopeptides synthesized by *in vitro* glycosylation can be used as a model system in the bioinfomatics field. A glycoprotein has a highly complex structure and, since it is hard to study the effect of glycosylation on 3-dimensional structures, the role of carbohydrates can be inferred from studying glycopeptides [14]. The transmission pattern of glycopeptides in the human body is different from that of native peptides, therefore, glycosylation could also be a new drug targeting method [12].

A eukaryotic system, such as a mammalian or insect cell system, is used for the industrial production of glycoproteins [13], and attempts have been made to modify their oligosaccharide structures toward a natural form using additives [2]. Although the oligosaccharide structures of glycoproteins are important from an immunopathological aspect [1], an intact form of a oligosaccharide is hard to obtain. However, the *in vitro* glycosylation of a protein produced in *E. coli* with a specific enzyme for the production of an O-GlcNAc-modified recombinant protein was recently attempted [10]. *In vitro* glycosylation promises to be a valuable tool for the production of pharmaceutical glycoproteins from non-glycosylated recombinant proteins produced in prokaryotic systems, as it produces a high yield and is cost effective. In a previous study by the current authors, the *in vitro* glycosylation of glucose oxidase was performed and a promising result obtained [9].

Accordingly, the current study was undertaken to expand this previous work on peptides and native proteins, and applied a more accurate technique, MALDI-TOF-MS, to analyze the glycosylation reaction. PNGase F belongs to the endoglycosidase family and releases an N-glycosylated residue from its glycoprotein. PNGase F is active when the peptide is longer than the trimer and the sugar residue is longer than *N,N'*-diacetyl chitobiose (GlcNAcβ1→4GlcNAc). Furthermore, the inner core must be GlcNAc [3, 4]. A deglycosylation enzyme, PNGase F (peptide-*N*-glycosidase F), was used to attach a sugar moiety to a peptide and protein using a reverse reaction in this study. The 1,4-

dioxane or ethylene glycol was used as the organic solvent to reduce the water activity [16] and shift the reaction equilibrium.

MATERIALS AND METHODS

Enzyme and Substrates

The PNGase F (peptide-*N*-glycosidase F, peptide-N^4 (*N*-acetyl-β-glucosaminyl) asparagine amidase F, EC 3.5.1.52) was purchased from Roche Applied Sciences, Mannheim, Germany. One unit was defined as the enzyme activity that hydrolyzed 1 nmol dansyl fetuin glycopeptide in 1 min at 37°C and pH 7.2. The pentapeptide (RKDVY), RNase A, *N,N'*-diacetyl chitobiose, and other reagents were all purchased from Sigma. The organic solvents were dehydrated using molecular sieves.

Amination of *N,N'*-Diacetyl Chitobiose

The amination method of *N,N'*-diacetyl chitobiose to *N,N'*-diacetyl chitobiosylamine developed by Likhosherstov was used [8]. Ammonium bicarbonate (NH_4HCO_3) was added to 1 M *N,N'*-diacetyl chitobiose solution to saturate the solution. The mixture was then incubated for ten days at room temperature, and ammonium bicarbonate was continuously added during the incubation to saturate the solution. TLC (thin layer chromatography) was used to analyze the amination. The reactant and product were separated by their respective mobilities, and the extent of the amination was determined using a colorimetric ninhydrin amine assay. The aminated chitobiose was quantified indirectly by measuring the remaining chitobiose. The calibration curve was obtained using a standard solution of *N,N'*-diacetyl chitobiose with a Total Lab. (Phoretix, U.S.A.). To remove the remaining ammonium bicarbonate, lyophilization was repeated six times.

Glycosylation of Pentapeptide (RKDVY)

Forty nmol of pentapeptide, 400 μmol of a sugar mixture, 3 units of PNGase F, and 10 μl of ethylene glycol were mixed, and then H_2O was added to make a final volume of 20 μl. The mixture was then incubated at 37°C for 11 h. A negative control reaction was also performed without the PNGase F. One and half μl of the solution was sampled every hour, then the aliquot was freeze-dried and stored at -70°C until analyzed.

Glycosylation of RNase A

One μg of RNase A, 200 μmol of a sugar mixture, 3 units of PNGase F, and 10 μl of 1,4-dioxane were mixed, then H_2O was added to make a final volume of 20 μl. The mixture was incubated at 37°C for 9 h, then the reaction was stopped by boiling, and the mixture stored at -70°C until analyzed.

MALDI-TOF-MS

The time-of-flight mass spectra of the peptide were obtained using a Voyager Biospectrometry workstation with a linear mass analyzer (Applied Biosystems, Framingham, MA, U.S.A.), which employed a 337 nm nitrogen laser and 1.2 m linear flight tube. The positive ion spectra were collected at a 30 kV accelerating voltage, and typically about 64 scans were averaged. The system was calibrated with an intact pentapeptide (MW: 679.8) and the calibration kit (Applied Biosystems). In the case of the protein, the spectra were obtained using a Voyager-DE™ Biospectrometry workstation (Applied Biosystems, Framingham, MA, U.S.A.) with a linear mode analyzer and 2.0 m flight tube.

To prepare the sample, first, 5 μl of 50 (v/v)% acetonitrile including 0.1% trifluoroacetic acid were added to the sample tube. Then, 1 μl of the prepared solution was mixed with 3 μl of a matrix solution (α-cyano-4-hydroxycinnamic acid, 10 mg/ml), and 1 μl of the solution was loaded on a gold plate and air-dried. In the case of RNase A, 1 μg of RNase in 3 μl of a matrix solution (sinapinic acid, 10 mg/ml) was used.

RESULTS AND DISCUSSION

Amination of *N,N'*-Diacetyl Chitobiose

Although PNGase F is classified as an endoglycosidase, it is one of the amidases that hydrolyzes the β-amide linkage connected to a side branch of an asparagine residue. During deglycosylation, asparagine is altered to aspartate (step 1 in Fig. 1), and subsequently, the amino-sugar group is spontaneously hydrolyzed into ammonia and an intact oligosaccharide (step 2 in Fig. 1). In our previous study, ammonium bicarbonate was used as the ammonia source that is essential in the reverse reaction in the step 2. However, it might be more efficient to use an aminated sugar than an ammonium salt, and the *N,N'*-diacetyl chitobiose was aminated prior to the glycosylation reaction. The extent of amination was quantified using TLC, as described in Materials and Methods. The R_f values on the TLC were found to be 0.49 for *N,N'*-diacetyl chitobiose and 0.38 for *N,N'*-diacetyl chitobiosylamine, and a linear calibration

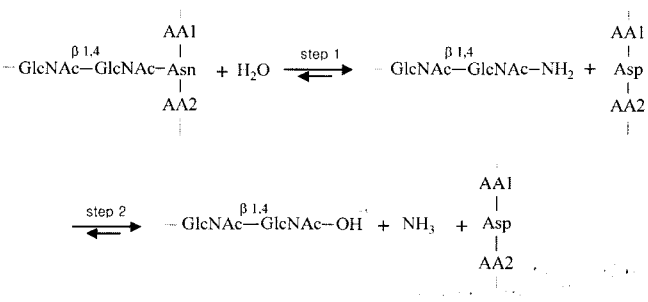

Fig. 1. Reaction mechanism of PNGase F.

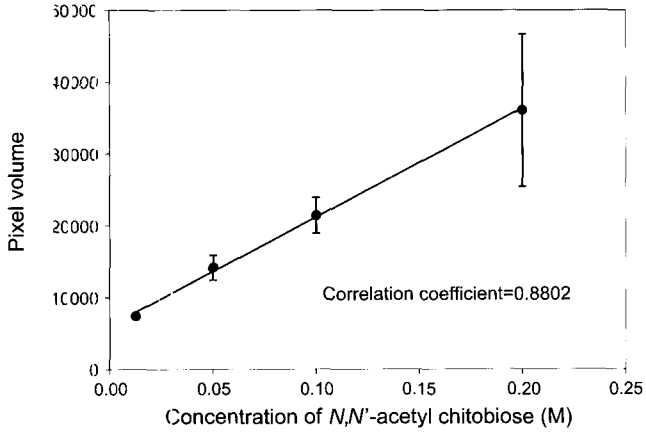

Fig. 2. Standard curve of *N,N'*-diacetyl chitobiose.

curve was obtained for *N,N'*-diacetyl chitobiose, as shown in Fig. 2. The extent of the amination of *N,N'*-diacetyl chitobiose to *N,N'*-diacetyl chitobiosylamine was 60%.

Glycosylation of Pentapeptide

In a glycosylation reaction, the change of molecular weight and the formation of glycoprotein can be detected by SDS-PAGE and carbohydrate-specific PAS (Periodic Acid Schiff) staining, respectively [9]. However, since a slight change in molecular weight is difficult to detect with SDS-PAGE, MALDI-TOF-MS was applied in this study as a more powerful technique to determine accurate molecular weight.

The model pentapeptide used for the glycosylation consisted of arginine, lysine, aspartate, valine, and tyrosine, and the molecular weight was 679.8. According to the reaction mechanism of PNGase F, glycosylation occurs on the aspartate residue and the molecular weight of the glycosylated pentapeptide is predicted to be 1,085.2. Since the glycosylation reaction is a condensation reaction, the molecular weight increases by 423.4 due to the *N,N'*-diacetyl chitobiosylamine and decreases by 18 due to the water molecule. Therefore, the net increase will be 405.4. The MALDI-TOF-MS results showed the existence of a glycosylated pentapeptide peak at around 1,090 (Fig. 3B). The measured molecular weight was slightly higher than the expected mass of 1,086.2 (increase by 1 due to protonation). This is due to an error coming from the extrapolation measurement in the MALDI-TOF-MS. A matrix molecule and pentapeptide were used to calibrate the equipment in this assay. The extrapolation of the glycosylated pentapeptide might have caused the deviation, since the peak of the glycosylated pentapeptide was located outside the calibration curve.

Contrary to the expectation, the glycosylated pentapeptide peak appeared also in the negative control without PNGase F (Fig. 3A), suggesting a nonenzymatic glycosylation reaction. Nonenzymatic glycosylation, known as the Maillard reaction, occurs on the arginine or lysine residue when the length of the sugar residue is one or two [5, 7]. The Maillard reaction takes place when reducing sugars are incubated with amino acids or proteins. It occurs in most foods on heating and in the human body. Since a sugar mixture containing both *N,N'*-acetyl chitobiose and chitobiosylamine was used, nonenzymatic glycosylation might have occurred between the *N,N'*-acetyl chitobiose and a lysine or arginine residue in the pentapeptide.

Although the glycosylated pentapeptide was also observed in the negative control, the peak intensity of the glycopeptide in the reaction mixture with PNGase F was much higher. While the peak intensities in one sample cannot be directly compared with those in other samples, a quantitative comparison among the components in one sample is reasonable [15]. Therefore, taking into account the other analytical conditions (laser intensity, sample concentration, grid voltage etc.), this increase in the peak intensity is

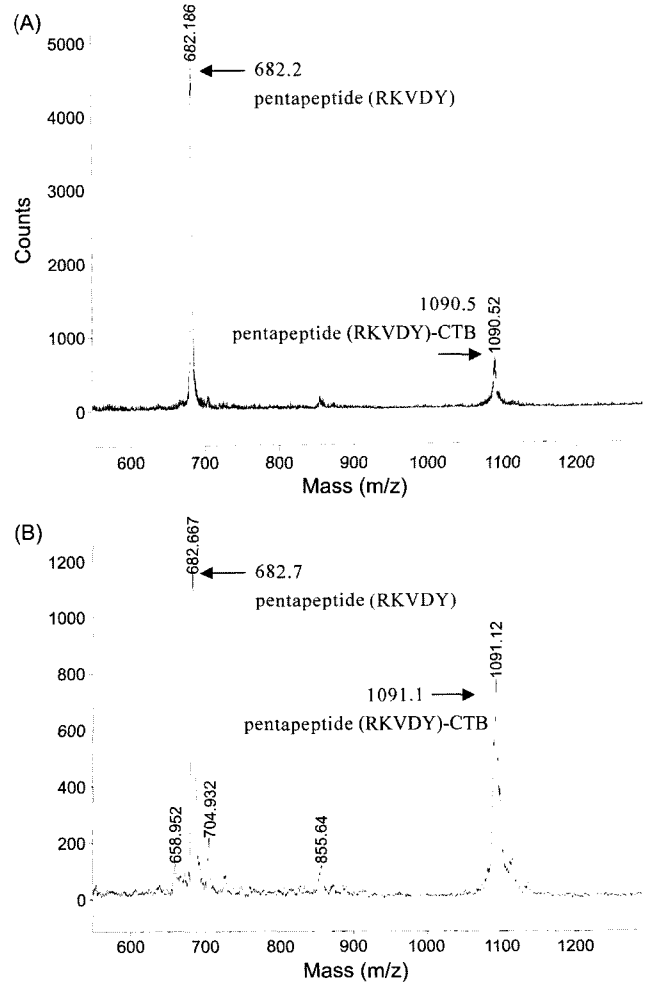

Fig. 3. *In vitro* glycosylation of pentapeptide in 50% (v/v) ethylene glycol (2 h reaction).
(a) Negative control (without PNGase F). (b) Glycosylation (with PNGase F).

regarded as an increase in the degree of glycosylation. This implies that only nonenzymatic glycosylation occurred in the reaction mixture without PNGase F, whereas both enzymatic and nonenzymatic glycosylation occurred in the reaction mixture with PNGase F. To quantify the glycosylation, the relative intensity of the glycopeptide was defined as follows:

$$\text{Relative intensity} = \frac{\text{intensity of glycopeptide}}{\text{intensity of peptide} + \text{intensity of glycopeptide}}$$

The relative intensity in the reaction either with or without PNGase F is shown in Fig. 4. The relative intensity of the enzymatic reaction was almost twice higher than that of the nonenzymatic reaction.

Glycosylation of RNase A

RNase is a relatively small protein and facilitative of a MALDI-TOF-MS analysis. RNase A is a non-glycosylated form of RNase, whose molecular weight is 13,688 Da, and the resulting molecular weight with glycosylation is 14,094 Da. In our previous study, a protein denatured by sodium dodecyl sulfate (SDS) was used. However, since the presence of SDS may affect the activity of PNGase F, a native form of RNase A was used in this study to eliminate the possible inhibition by the detergent. The result of RNase A glycosylation with *N,N'*-diacetyl chitobiosylamine in 50% (v/v) 1,4-dioxane after 9 h of incubation is shown in Fig. 5. Similar to the peptide, a glycoprotein was observed in both reaction mixtures with and without PNGase F. However, the product peak of the reaction mixture with PNGase F was much higher than that without PNGase F. It is worth to note that another peak representing a compound containing two *N,N'*-diacetyl chitobiosylamines was observed in the reaction mixture with PNGase F,

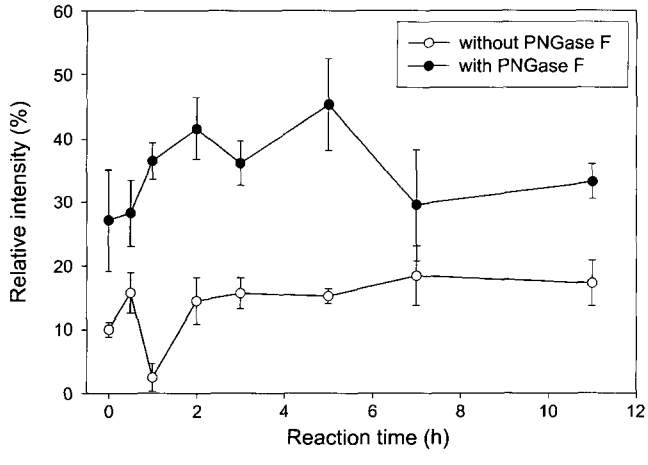

Fig. 4. Relative intensity of glycosylation of pentapeptide in 50% (v/v) ethylene glycol.

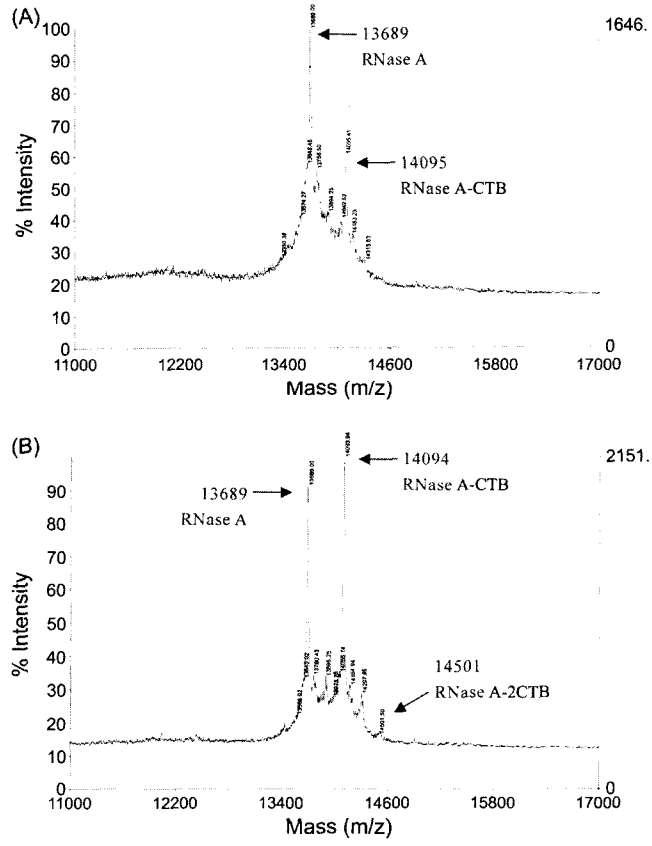

Fig. 5. Glycosylation of RNase A by PNGase F in 50% (v/v) 1,4-dioxane (9 h reaction).
(a) Negative control (without PNGase F). (b) Glycosylation (with PNGase F).

and its molecular weight was 14,501 Da. This could be interpreted in two ways either the glycosylation occurred in two different sites, or a dimer form of *N,N'*-diacetyl chitobiosylamine was attached to one site. Further investigation is required to elucidate the property of the peak representing a compound with two *N,N'*-diacetyl chitobiosylamines.

The *in vitro* glycosylation of pentapeptide (RKDVY) and RNase A using PNGase F was successfully performed at 37°C in 50% (v/v) 1,4-dioxane or ethylene glycol. Although nonenzymatic glycosylation, known as the Maillard reaction, occurred, the extent of glycosylation was much higher in the enzymatic reaction. Therefore, it is quite possible that non-glycosylated proteins expressed in a prokaryotic system can be *in vitro* glycosylated using PNGase F.

Acknowledgment

The authors wish to acknowledge the financial support of LG Life Science and the Nano Bio-Electronic & System Center.

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