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Original article

Recombinat Production and Characterization of the SUMO-Tagged N-Glycosidase F Enzyme

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Abstract

Glycans, a key component of cells, are abundant and diverse biopolymers found in covalent bonds of sucrose attached to proteins and lipids. They significantly contribute to mass and structural variation in biological systems. In order to characterize glycans, study their effects in various experiments, and comprehend their roles, it is necessary to isolate them from the proteins with which they are associated. Chemical methods and various enzymes separate glycans. The ease of application of chemical methods, despite their low cost, chemical methods ease of application affects the chemical structure of both glycans and the remaining part of the polypeptide during deglycosylation. Additionally, the high salt content of the separated glycans makes mass spectrometry analysis of these glycants difficult. For these reasons, the use of enzymes in glycan studies has increased in recent years. One of the most commonly used enzymes in this field, *N*-glycosidase F has a wide spectrum and the ability to successfully release various *N*-glycan structures from glycoprotein.

In this study, the PNGase F enzyme, secreted by *Flavobacterium meningosepticum*, was efficiently produced in a recombinant manner. The enzyme, which contains 314 amino acids, is the most effective method for removing N-glycan from glycoproteins.

Keywords: *N*-linked glycans, Glycoproteins, Glycosidases, Deglycosylation, *In vivo* cloning.

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INTRODUCTION

Enzymatic glycosylation is a prevalent process that modifies proteins during and after their synthesis. It plays a crucial role in controlling the structure, solubility, folding, and stability of proteins (Duman et al., 2021; Pekdemir et al., 2022). The oligosaccharide (glycan) component of glycoproteins possesses a diverse range of biological functions and regulates numerous interactions among cells and molecules. Protein glycosylation varies among individuals but remains highly stable within each individual under normal physiological conditions (Karav et al., 2016). Nevertheless, detectable alterations in glycosylation are evident in various clinical states including cancer, autoimmune disorders, and inflammatory illnesses, as well as during the natural aging process (Varki, 1993). Therefore, the examination of protein glycosylation has been garnering attention in clinical and fundamental research and holds great significance in the pharmaceutical and biotechnology sectors (Krištić et al., 2014).

Glycans, which are carbohydrate-based polymers, are essential components of biological systems and may be found in various living creatures such as plants, animals, and microbial sources. Covalent connections between saccharides and proteins or lipids exist, exerting an influence on structural and mass changes. Glycobiology is a multidisciplinary area of study that investigates the intricate structures, chemical characteristics, biosynthesis processes, and biological functions of glycans (Bunyatratchata et al., 2023). Protein glycosylation is a key process that encompasses three primary categories: *N*-glycans, O-glycans, and glycosaminoglycans (proteoglycans). *O*-glycans establish covalent linkages with serine or threonine residues, whereas *N*-glycans bind to particular asparagine residues through *N*-acetylglucosamine bonds (HexNAc). The fundamental composition of *N*-glycans comprises of two HexNAc and three mannose residues, occasionally accommodating fucose. The initiation of *N*-glycan production occurs in the endoplasmic reticulum and is subsequently elongated by additional monosaccharides, regulated by glycosyltransferases and glycosidases (Şahutoğlu et al., 2020; Varki, 1993).

In the last ten years, there have been significant advancements in procedures that allow for the structural characterization of glycans (Karav et al., 2015; Parc et al., 2017). Peptide *N*-glycosidase F (PNGase F; EC 3.5.1.52) is the enzyme most commonly used in glycoanalytical workflows to remove *N*-glycans from glycoproteins. It is preferred because of its wide specificity and ability to release a diverse range of *N*-glycan structures from glycoproteins. This enzyme was first described by Plummer et al. in 1984 and later by Freeze and Varki in 1986 (Freeze & Varki, 1986). PNGase F is a very effective enzyme that can break down a wide range of substances. It specifically breaks the glycosylamine bond, resulting in the production of a peptide without any carbohydrates and an intact oligosaccharide that contains a di-acetylchitobiose unit at the end where the bond was broken (Tarentino et al., 1985). The initial attempts to isolate PNGase F demonstrated its strong enzymatic activity, which eventually resulted in its successful purification from many species. Each species showed promising levels of

effectiveness (Barsomian et al., 1990). *Flavobacterium meningosepticum's* naturally secreted PNGase F was isolated from bacterial cultures using complex methods, yielding small amounts of pure protein, highlighting the challenges in purification (Mussar et al., 1989). This isolated PNGase F is characterized by a molecular weight of approximately 35 kDa and consists of a polypeptide chain that spans 314 amino acids (Loo et al., 2002). In addition, the enzymatic activity of the substance reaches its highest point at a pH of 8.5, which emphasizes its ideal biochemical conditions for proper functioning (Tarentino et al., 1985).

The implementation of advanced techniques in cloning approaches has greatly improved the efficiency of producing and purifying clones inside *Escherichia coli* (Barsomian et al., 1990). In addition, research undertaken by Loo et al. has clarified that the removal of the enzyme's signal sequence leads to improved efficiency, demonstrating ongoing progress in boosting its enzymatic effectiveness (Loo et al., 2002). The use of PNGase F is unparalleled as the leading technique for completely removing almost all *N*-glycans from glycoproteins, except in cases where the glycan does not have a core α(1 → 3)-fucose. In such cases, PNGase F can effectively release a wide range of asparagine-linked complex, hybrid, or high-mannose oligosaccharides (Tarentino et al., 1985).

This article showcases a straightforward and reliable method for producing PNGase F with a high yield (51%) and a high level of purity (>90%). Research on the substrate specificity of *N*-glycosidases using glycoproteins shows that PNGase F is a very efficient enzyme for releasing *N*-glycans, making it valuable for investigations in the field of glycobiology.

MATERIALS and METHODS

The Dissolving of Genomic DNA

The DNA of Elizabethkingia miricola ATCC 33958 is obtained from the American Type Culture Collection (ATCC). According to the manufacturer's instructions, the genomic DNA was dissolved in 250 µL of pure water and incubated at 37°C for 1 hour. It was then incubated for a night in a 4°C incubator, and the next day it was prepared for use at 60°C for an hour, so that the entire DNA could be thoroughly dissolved.

Gene cloning, expression, and purification

PNGase F is primarily derived from the NCBI database of the amino acid and nucleotide series of this gene for the design of the primers necessary for the reproduction of the genome region of the enzyme. The primary design has been carried out taking into account all the primary rules of design. The lyofilized primary sets were dissolved by pipeting with the company's proposed amount of pure water, and 100 µM stocks were obtained. 100 µM primersets are diluted and 10 µM stocks are prepared for PCR use. 100 µM stocks are stored at -80°C.

The gene cloning procedure was carried out using the Expresso® Rhamnose Cloning & Protein Expression *in vivo* system (Lucigen Corp., Middleton, WI, United States), following the instructions provided by the manufacturer. The coding sequence of PNGase F was amplified using suitable cloning primers and subsequently inserted into the pRham™ N-His Sumo Kan vector via cloning. The enzyme was replicated by introducing an N-terminal polyhistidine Sumo tag. Poly-histidine-SUMO tagged PNGase F was synthesized in the E. coli host using LB media. The yield obtained was around 1.7 mg per liter. The ideal induction conditions involved a final concentration of 0.2% L-rhamnose and incubation at 37°C for 18-24 hours. The protein was purified using Ni-charged resins (Thermo Fisher Scientific, Waltham, MA, USA) after bacterial lysis. The bound protein was eluted using a solution of 250 mM imidazole, resulting in a high level of purity. The concentration of the enzyme was measured using a Qubit Protein Assay Kit (Life Technologies, Grand Island, NY, USA) and seen using SDS-PAGE (4-12%). The PNGase F that was created through genetic recombination was stored at a temperature of -80°C for future investigation (Karav et al., 2016; Sucu et al., 2021).

Testing the activity of the produced recombinant enzyme and determining the optimal reaction conditions

The activity of the produced recombinant enzyme was tested with the RNase B model glycoprotein. RNase B has a molecular weight of 17 kDa and has only one *N*-glycosylation region. As a result of deglycosylation, the molecular weight of RNase B decreases from 17 kDa to 14 kDa. This difference is a distinctive change in SDS-PAGE gel electrophoresis. In order to test the enzyme's activity, 1 µL of RNase B model glycoprotein, 1µL glycoproteine denaturation buffer solution (10X) and 1µl of pure water were initially added to an ependorphic tube, and 5 minutes of denaturing at 95°C was achieved. Subsequently, 2 µL of Glycobuffer 2 (10X) was supplemented with the PNGase F enzyme produced as a detergent for the release of proteins. Instead of the enzyme, 6 µL of pure water was added to the control tube and incubated for 3 hours at 37 °C to a total volume of 20 µL. The incubated specimens were mixed 1:1 with the 20 µL Laemmli Sample Buffer (2X) and incubated for 5 minutes at 95°C.

Observations have been made on the activity of the recombinant enzyme at various pH levels and temperatures in order to identify the most favorable operating conditions. The RNase B protein was utilized to activate the enzyme, which was examined individually at pH 5, pH 8, and pH 10, with temperatures set at 37°C, 30°C, and 24°C, respectively. To assess the enzyme's activity under different pH and temperature conditions, 1 µL of the RNase B model glycoprotein was initially placed in an ependorphic tube. Subsequently, 1 µL of the glycoprotein denaturation buffer solution (10X) and 1 µL of distilled water were added, and the mixture was denatured at 95°C for 5 minutes. Next, a 0.2 M sodium phosphate buffer solution was introduced within the desired pH range. Subsequently, 2 µL of a 10% NP-40 solution, 1 µL of clean water, and 5 µL of recombinant PNGase F enzyme were added as

detergents. The kinetic parameters were measured by determining the calorimetric quantities of glycan cut after 20 minutes in the linear region. This was done by reacting lactoferrin under optimum circumstances at 8 different concentrations ranging from 0.1 to 0.8 mg/mL. The V_{max} and K_{cat} values were then computed. The concentration of the enzyme was 0.025 mg/mL (Pekdemir et al., 2022).

RESULTS and DISCUSSION

The enzyme, generated by our *in vivo* cloning approach, has a significant advantage over previously employed methods described in the literature. It can be created rapidly, resulting in time savings and facilitating the implementation of novel enzyme production strategies required in the present day. The *in vivo* cloning approach involves the immediate cloning of PCR products without the need for enzymatic processes. This procedure occurs totally within the host cell, allowing the host bacterium to utilize its own enzymes, eliminating the need for external use of any restriction enzyme (Sucu et al., 2021).

The efficacy of the deglycosylation procedure and the functionality of the enzyme were verified by assessing its activity on the model protein, RNase B. Upon analyzing the gel pictures, it was noted that the recombinant enzyme exhibits its highest level of activity at a pH of 5 and a temperature of 30°C. It also demonstrates activity near to its maximal level at a pH of 10 and a temperature of 24°C. Multiple investigations have demonstrated that the PNGase F enzyme exhibits its maximum efficiency at a pH of 8.5 and a temperature of 37 °C. However, it is established that the enzyme retains at least 80% of its activity within the pH range of 7.5 to 9.5 (Loo et al., 2002; Nuck et al., 1990). However, it is also probable that this observed activity is a result of the N-His SUMO label used throughout the cloning process. Due to the absence of prior cloning of the PNGase F enzyme with this label, it is hypothesized that the N-His SUMO label may have influenced the enzymatic activity conditions. Nevertheless, as the enzyme's efficacy in the deglycosylation process remains unaltered and is exceptionally potent in both acidic and basic conditions, it is believed to not present any issues for the investigation. Conversely, the enzyme that gets produced has the capacity to be utilized with samples that are sensitive to either acid or base. Additional investigation is required to uncover the unknown impacts of the N-His SUMO label on the enzyme.

The outcome of our investigation yielded 0.88 milligrams of PNGase F enzyme from a volume of 1 liter of culture. Based on previous research on recombinant bacterial PNGase F, the initial purification experiments yielded 0.1-0.5 milligram per 1 L of culture (Mussar et al., 1989). Due to later improvements, Loo and his coworkers succeeded in producing 8 mg of the enzyme, achieving a high yield from a 1 L culture (Loo et al., 2002). However, throughout the examination of these research, a multitude of exogenous enzymes were employed in the manipulation of PCR products and during the various stages of cloning. The utilization of these enzymes is expensive, as it is also time-consuming. Thus, in contrast to the other cloning technologies examined in our work, there is no need for enzymatic

processing or purification of the PCR result. Serial connections are not limited by the absence of limiting enzymes. Research conducted to validate the enzyme activity of the RNase B protein has demonstrated that the recombinantly produced enzyme exhibits its highest level of activity at a pH of 5 and a temperature of 30°C. It was subsequently observed that the activity peaked at a pH of 10 and a temperature of 24°C, approaching its maximum level. The lowest detected activity was documented at a pH of 8 and a temperature of 30°C, which differs from earlier experiments reported in the literature. The results of several pH and temperature combinations are depicted in Figure 1 and Figure 2.

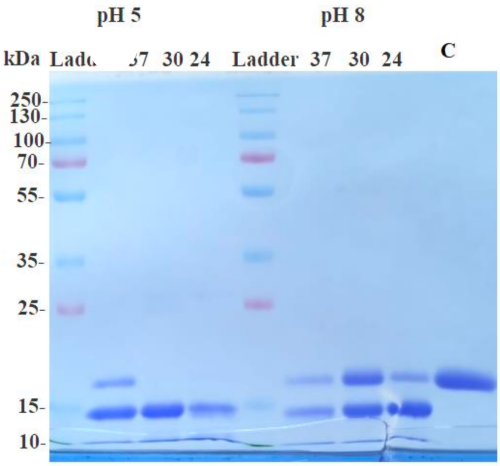

Figure 1. Determination of optimal working conditions for the PNGase F enzyme at pH 5 and pH 8 at 37°C, 30°C and 24°C.

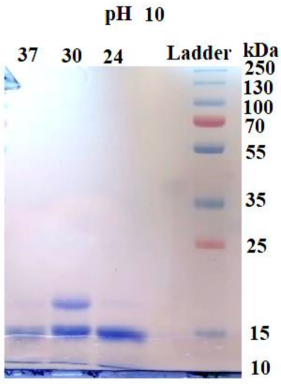

Figure 2. Determination of optimal working conditions for the PNGase F enzyme at pH10 at 37°C, 30°C and 24°C.

The enzyme concentration employed for determining ideal conditions is maintained at a low level, while the focus is on achieving the desired partial deglucosylation time. Consequently, there is an absence of enzyme bands in the displayed gels. However, the activity can be verified by Rnase B. According to the non-linear regression analysis, K_m =0.2524 mg/mL and *Vmax* =0.0038ml/mLxmin

were detected. Linear regression results were identified as K_m=0.5252 mg/mL and *Vmax*=0.0055 ml/mLxmin.

This study has contributed to filling the gaps in the literature on the production methods of enzymes used in *N*-glycan separation, improving time-consuming methods, and developing new ideas on enzyme production.

Conclusion

To understand the properties of glycans, which play a crucial role in biological systems, it is necessary to separate them from proteins. This can be achieved using several deglycosylation techniques. Chemical processes provide significant power but can raise concerns regarding the environment and safety. As a result, enzymatic approaches, namely those involving PNGases, are an attractive alternative. PNGase F is renowned for its precision in liberating *N*-glycan structures from glycoproteins. Nevertheless, obstacles in the process of purification continue to hinder the possibility of producing on a big scale. Despite the presence of purification challenges, improvements in cloning techniques and the elimination of signal sequences have enhanced the efficiency of production processes. The tool's exceptional capability to extract almost all *N*-glycans makes it indispensable for glycoprotein analysis. In order to facilitate large-scale production of PNGase F, future research should focus on enhancing the purification process. Exploring the potential of alternative deglycosylating enzymes and improving enzymatic processes could provide viable substitutes.

Hence, to achieve efficient glycan characterisation, one must address the challenges associated with enzymatic approaches, particularly in the purification of PNGase F. Additionally, it is necessary to investigate the functions and applications of glycans. The incorporation of other disciplines is essential in order to fully exploit the potential of glycans in diverse domains, such as healthcare and biotechnology.

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