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Effects of *N*-glycosylation on the biochemical properties of recombinant bEK_L expressed in *Pichia pastoris*

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ABSTRACT

Enterokinase is an ideal tool protease for cleaving fusion proteins in genetic engineering. The bovine enterokinase light chain (bEK_L) produced in *Pichia pastoris* was shown to be a glycoprotein. To study the effects of *N*-glycosylation on the biochemical properties of bEK_L, the enzyme was deglycosylated via site-directed mutagenesis. The results showed that elimination of the *N*-glycosylation sites of bEK_L (N64, N103 and N165) did not significantly affect the protein secretion level in *P. pastoris*, but it does greatly influence its enzymatic activity. The N64Q increased the specific activity of the enzyme for GD_4K-β-naphthylamide and improved its catalytic efficiency. Moreover, the glycosylated bEK_L is more thermostable than its deglycosylated counterparts. Structural analysis of glycosylated and deglycosylated bEK_L revealed that the removal of *N*-glycosylation did not have pronounced changes on the secondary structure but there was a significant difference in the tertiary structure. In conclusion, this study demonstrated that the effects of glycosylation at different degrees and sites in bEK_L were diverse. Moreover, this work will provide theoretical support for designing enzymes on the basis of *N*-glycosylation to meet the demands of the biochemical industry.

1. Introduction

Glycosylation is one of the most complicated and ubiquitous post-translational modifications that may affect the function and structure of enzyme in all eukaryotes. More than half of all proteins found in nature are glycosylated, and three quarters of them contain *N*-glycosylation [1]. *N*-glycosylation often occurs at the asparagine residues within the consensus sequence Asn-X-Ser/Thr [2,3], where X cannot be proline, because the pyrrole ring structure of proline increases the rigidity of the peptide chain and inhibits glycosylation. Recent research has indicated that approximately 70%–90% of the consensus sequence is glycosylated [4]. The hydroxyl groups of threonine and/or serine residues in proteins can lead to the emergence of *O*-glycosylation [5,6]; however, little is known about the consensus sequence or structural characteristics of *O*-glycosylation.

N-glycosylation stems from the endoplasmic reticulum (ER). In a co-translational event, the oligosaccharide ($Glc_3Man_9GlcNAc_2$) that assembled on dolichyl-pyrophosphate is transferred to the appropriate asparagine residues of the protein [7,8]. This process is highly conserved in eukaryotes. Then, through a series of complicated processes, the oligosaccharide can develop into three forms based on the eukaryotic cell type, with the high-mannose type in yeasts and complex/hybrid type in higher eukaryotic cells (plants and animals) [9]. *N*-glycosylation means a great deal to many cellular processes, including protein secretion [10,11], protein folding [12], intracellular trafficking [13], and cell communication. Studies show that it is also involved in signal transduction and receptor activation [14]. Furthermore, *N*-glycosylation can affect protein stability [15,16], activity [17], and specificity[18].

Enterokinase (EC 3.4.21.9), a type II serine protease found in the duodenum, can activate trypsinogen via cleavage after the N-terminal hexapeptide Val-$(Asp)_4$-Lys without any undesirable amino acid residues on their amino termini [19]. The high specificity of enterokinase makes it an ideal protease in the cleavage of fusion proteins. The bovine enterokinase is composed of a heavy chain and a light chain connected by a disulfide bond; the heavy chain influences the recognition of a macromolecular substrate, and it also impacts inhibitor specificity and membrane association. The light chain, highly specific to the sequence $(Asp)_4$-Lys, is a catalytic subunit responsible for peptidase activity [20,21]. The analysis of amino acid sequences indicated that the bovine enterokinase light chain (bEK_L) contains three putative *N*-glycosylation sites at N64, N103, and N165 [22]. It has been confirmed that N-linked

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glycan is critical to the structure and function of glycoprotein; however, there is almost no information available to explore the effects of *N*-glycosylation on the biochemical properties of bEK_L.

To explore the effects of N-linked glycan on the enzymatic characteristics of recombinant bEK_L expressed in *Pichia pastoris*, we constructed a series of glycosylation-deficient mutants by Quick Change site-directed mutagenesis, in which the asparagine (N) residue of the potential *N*-glycosylation sites in bEK_L was substituted with the structurally similar glutamine (Q) residue; the significant changes that occurred after deglycosylation were subsequently examined. The roles of *N*-glycosylation on enzyme characteristics (e.g, molecular mass, secretion, activity, thermostability and kinetic parameters) were discussed.

2. Materials and methods

2.1. Strains, reagents, and medium

The plasmid pPIC9K and *P. pastoris* strain GS115 were obtained from Invitrogen (Thermo-Fisher Scientific, Waltham, MA, USA). The *Escherichia coli* Top10 and fusion protein DsbC-VHH were reserved in our laboratory. Phusion High-Fidelity DNA Polymerases were purchased from Thermo-Fisher Scientific, while the restriction enzyme *Sal*I was obtained from Takara (People's Republic of China), endoglycosidase H (*Endo* H) from NEB (Cambridge, MA, USA), and the substrate Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide (GD_4K-na) from Sigma-Aldrich Co. (St. Louis, MO, USA). All remaining reagents used in the experiments were analytically pure.

2.2. Construction of the expression vector and deglycosylation mutants

The genetic sequence of the bEK_L (GeneBank ID: P98072) was synthesized by Sangon Biotech (People's Republic of China) and cloned into the expression vector pPIC9K to acquire the plasmid pPIC9K-bEK_L. To construct the bEK_L deglycosylated mutants in which one or more N-linked glycan chains were removed, the codon for Asn (N) was substituted by Gln (Q) at N64, N103, and N165 using the modified Quick Change method [23]. The recombinant plasmid was linearized by *Sal*I and then electrotransformed into GS115 through electroporation by a Bio-Rad Micropulser Electroporater (Bio-Rad, Hercules, CA, USA). The mutagenic primers (synthesized by GENEWIZ Inc.) used in this study are listed in Table 1.

2.3. Expression and purification of bEK_L proteins in P. pastoris

The positive clones were inoculated in 50 mL of BMGY medium and cultured at 30 °C with 250 rpm shaking speed until the OD_{600} reached 1–3. Then, the cells were collected, resuspended to a final OD_{600} of 1.0 in 100 mL of BMMY medium, and grown in shaking flasks at 30 °C while adding 100% methanol to reach a final concentration of 1% methanol every 24 h. After 96 h, the culture was centrifuged at 10,000 rpm for 20 min and the supernatant containing the secreted protein was harvested.

The supernatant was filtered through a 0.22 μm filter and then dialyzed overnight in equilibration buffer (20 mM sodium phosphate, 0.3 M NaCl, pH 8.0). The dialysate was loaded onto an Ni^{2+} affinity column (1 cm × 5 cm; GE Healthcare, Uppsala, Sweden) equilibrated with equilibration buffer. The column was washed with wash buffer (20 mM sodium phosphate, 0.3 M NaCl, 40 mM imidazole, pH 8.0) and eluted with elution buffer (20 mM sodium phosphate, 0.3 M NaCl, 300 mM imidazole, pH 8.0). The elution collection was dialyzed overnight in 20 mM Tris-HCl (pH 8.0) and concentrated by ultrafiltration on Amicon Uitra-15 Centrifugal Filter Units (Millipore Corporation, Darmstadt, Germany) with a molecular weight cutoff (MWCO) of 10 kDa. Then, 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out to verify the protein.

Table 1
Primers for the site mutation.

Mutations	Sequence (5′ to 3′)[a]
N64Q	CAACTTACAAGTCCACAAATTGAAACTAGACT(forward) TGGACTTGTAAGTTGAGAGGCCATATG (reverse)
N103Q	CAATACACAGACTACATCCAACCAATATGTT(forward) GTAGTCTGTGTATTGAACTTTCATCTCCAAG (reverse)
N165Q	CAAATCACTGAAAACATGGTTTGTGCTGGT(forward) GTTTTCAGTGATTTGGTATTCTGGCATTTGTTG (reverse)

[a] Nucleotides that were changed to encode Gln are underlined.

2.4. Deglycosylation of glycosylated bEK_L by Endo H

Endo H (NEB) was used to cleave the N-linked glycan chains of the recombinant bEK_L. The glycoprotein samples (20 μg) were boiled for 10 min in denaturing buffer (0.4 M DTT and 5% SDS) to completely expose all N-linked oligosaccharide chains; then, deglycosylation was carried out by treatment with 1000 units of *Endo* H in GlycoBuffer (50 mM of sodium acetate, pH 6.0) for 1 h at 37 °C. Samples were subsequently analyzed by SDS-PAGE.

2.5. Determination of enzymatic activity and protein concentration

Enzyme activity of was estimated with the cleavage of the synthetic peptide substrate, GD_4K-na, as described previously [24,25]. Given the high specificity of bEK_L to the sequence $(Asp)_4$-Lys, the β-naphthylamide was released and fluoresced after bEK_L was added into GD_4K-na. Fluorescence was measured by excitation at 337 nm and emission at 420 nm using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Enzymatic activity was measured as the change in fluorescence intensity with time. One unit (U) was defined as one absorbance increase of fluorescence in 1 min (1U = 1 abs/min) under 0.05 mM GD_4K-na. Lineweaver-Burk plot analysis was used to calculate the kinetic parameters, K_m and k_{cat}.

Enzyme activity was also detected by the digestion of the fusion protein DsbC-VHH harboring the bEK_L recognition sequence, $(Asp)_4$-Lys. Moreover, 2 μg of bEK_L was added to the fusion protein dissolved in 20 mM Tris-HCl (pH 8.0) and then incubated at 25 °C for 8 h. The samples were subsequently analyzed by SDS-PAGE.

Proteins concentrations were estimated at 280 nm with a NanoDrop ND-2000 spectrophotometer (Thermo-Fisher Scientific).

2.6. Optimal temperature, pH, and thermostability measurement of bEK_L

The enzyme was dialyzed overnight in different buffers such as sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0–9.0), and sodium hydrogen phosphate-NaOH (pH 10.0–12.0) at 20 mM concentrations. Then the enzyme activity at different pH levels was assayed. The optimal temperature (T_{opt}) was obtained by measuring the enzyme activity at different temperatures ranging from 25 °C–65 °C at intervals of 5 °C. The optimal activity was taken as a control (100%).

To study the thermostability of bEK_L, the enzyme was preincubated at 50 °C for varying periods ranging from 0 to 180 min and then cooled on ice for 5 min. The enzyme activity was measured at room temperature (25 °C) and the remaining activity was recorded as a percentage of the original activity. The enzyme kinetic behavior of bEK_L was calculated by plotting the natural logarithm of relative enzyme activity with time. The half-lives of the bEK_L variants at 50 °C were calculated using the following equation: $T_{1/2} = \ln 2/k_d$.

2.7. Far-ultraviolet (UV) circular dichroism (CD) spectroscopy

Far-UV CD spectra of 0.2 mg/mL of protein in 20 mM Tris-HCl (pH 8.0) were measured at 25 °C in the range from 190 to 260 nm with a 1 mm pathlength quartz cell using a Jasco-810 spectropolarimeter