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The role of N-glycosylation sites in the activity, stability, and expression of the recombinant elastase expressed by *Pichia pastoris*

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ABSTRACT

The *Pseudomonas aeruginosa* elastase (PAE), produced by *Pseudomonas aeruginosa* (*P. aeruginosa*), is a promising biocatalyst for peptide synthesis in organic solvents. As *P. aeruginosa* is an opportunistic pathogen, the enzyme has been heterologously over-expressed in the safe and efficient host, *Pichia pastoris* (*P. pastoris*) for its industrial application. The recombinant elastase (rPAE) contains three potential N-glycosylation sites (Asn-Xaa-Ser/Thr consensus sequences), and is heterogeneously N-glycosylated. To investigate the role of N-glycosylation in the activity, stability, and expression of rPAE, these potential N-glycosylation sites (N43, N212, and N280) were mutated using site-directed mutagenesis. Specifically the asparagine (Asn, N) residues were converted to glutamine (Gln, Q). The enzymatic activity and stability of non-glycosylated and glycosylated rPAE were then compared. The results indicated that the influence of N-glycosylation on its activity was insignificant. The non- and glycosylated isoforms of rPAE displayed similar kinetic parameters for hydrolyzing casein in aqueous medium, and when catalyzing bipeptide synthesis in 50% (v/v) DMSO, they exhibited identical substrate specificity and activity, and produced similar yields. However, N-glycosylation improved rPAE stability both in aqueous medium and in 50% (v/v) organic solvents. The half-lives of the glycosylated and non-glycosylated forms of rPAE at 70 °C were 32.2 and 23.1 min, respectively. Mutation of any potential N-glycosylation site was detrimental to its expression in *P. pastoris*. There was a 23.9% decrease in expression of the N43Q mutant, 63.6% of the N212Q mutant, and 63.7% of the N280Q mutant compared with the wild type. Furthermore, combined mutation of these sites resulted in an additional decrease in the caseinolytic activities of the mutants. These results indicated that all of the N-glycosylation sites were necessary for high-level expression of rPAE.

1. Introduction

The *Pseudomonas aeruginosa* elastase (PAE), encoded by the *las*B gene, is also called pseudolysin (EC 3.4.24.26). It is synthesized in the cytoplasm of *P. aeruginosa* as a pre-proenzyme of 498 amino acids (53.6 kDa), consisting of a 2.4-kDa signal peptide (23 amino acids), an 18.1-kDa pro-peptide (174 residues) and the 33.1-kDa mature protein (301 amino acids) [1]. The signal peptide is cleaved from the pre-proenzyme during translocation across the inner membrane, leaving a 51.2-kDa proenzyme (consisting of the pro-peptide and the mature protein). In the periplasm, the proenzyme is folded, guided by the pro-peptide, and a disulfide bond between Cys270 and Cys297 is formed. The pro-peptide is then removed by auto-proteolysis, but remains non-covalently attached to mature pseudolysin. A second disulfide bond between Cys30 and Cys58 of the enzyme is then formed. The pro-peptide and mature enzyme are secreted from the cell together, where they dissociate, and the liberated pro-peptide is degraded by the active enzyme [2].

PAE was crystallized, and its three-dimensional structure was assessed. It is a metalloprotease that requires a zinc atom, bound to His-140, His-144, and Glu-164, for its activity [2,3]. A calcium ion is also required for enzyme activity, and stabilizes its tertiary structure. Contact with the calcium ion is made by the carboxyl groups of Asp-136, Glu-172, Glu-175, and Asp-183, the carbonyl group of Leu-185, and one water molecule. The first and second disulfide bonds are essential for the stability and activity of the enzyme, respectively [3].

PAE is a well-characterized enzyme. It has a pI of 5.9, and optimal activity at a pH and temperature of 8 and 60 °C, respectively [4,5]. It is stable at pHs between 6.0 and 9.5, and when the temperature is below 60 °C [4,5]. The purified enzyme can hydrolyze a broad

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range of protein substrates, including feather, collagen, gelatin, and casein, while its actions are strongly inhibited by EDTA [4,5]. The protein has excellent stability and high catalytic activity in organic solvents, and produces considerably high equilibrium yields for peptide synthesis [6–9], making it a promising biocatalyst in this field.

P. aeruginosa is an opportunistic pathogen and causes severe nosocomial and community-acquired infections at various body sites [10,11]. Thus, the enzyme should be heterologously expressed in a safe and efficient host for industrial application. Due to advances in genetics and molecular biology, many targeted genes have been cloned and expressed in various eukaryotic and prokaryotic hosts to facilitate heterologous protein production.

The *las*B gene was previously expressed in an *Escherichia coli* system [12]. However, the recombinant protein was not secreted into the extracellular medium, and was thus only released upon cell lysis. Furthermore, this system only gave low expression levels, and was unable to meet the demands of industrial applications. Regarding eukaryotic host organisms, *Pichia pastoris* (*P. pastoris*), a methylotrophic yeast, is an excellent expression system that has been successfully used for over 20 years in both laboratory and industrial settings [13–15]. The attractiveness of *P. pastoris* as a host organism for heterologous protein production is due to its unique features, such as the availability of simple techniques for genetic modifications, simplified heterologous secreted protein purification processes, eukaryotic post-translational modifications, signal sequences and proteolytic processing, no secretion of endotoxins, and high-level expression of heterologous proteins, both intracellularly and extracellularly [16,17]. It may therefore be feasible to express PAE in *P. pastoris* and hence facilitate its industrial application. In a previous study, the full-length *las*B gene (with the native signal sequence) was cloned in to pPIC3.5K, and expressed in *P. pastoris* KM71. Recombinant protein was secreted into the culture medium at a concentration of approximately 0.45 g/l [18]. In our laboratory, *las*B (GenBank Accession No. JX003619) inserted in pPIC9K was successfully expressed in *P. pastoris* KM71, and the recombinant elastase (rPAE) was secreted and heterogeneously glycosylated [19].

N-glycosylation is one of the most common forms of protein post-translational modification in *P. pastoris*; approximately 70–90% asparagine (Asn, N) residues that form potential N-glycosylation sites (Asn-Xaa-Ser/Thr) are N-glycosylated. Furthermore, it was widely reported that N-glycosylation often plays a crucial role in the secretion, stability, and activity of heterologous proteins [20]. The amino acid sequence of PAE contains three potential N-glycosylation sites (N43, N212, and N280), and the rPAE expressed by *P. pastoris* was heterogeneously N-glycosylated [19]. It is important to study the effects of N-glycosylation on the activity, stability, and expression of *P. pastoris*-expressed rPAE. The most common method of studying the effects of N-glycosylation is using protein deglycosylation, which is typically achieved using site-directed interconversion of the Asn residue of the Asn-Xaa-Ser/Thr consensus sequence to the structurally related glutamine (Gln, Q) residue, or to other amino acids such as alanine (Ala, A) [20]. In this paper, the three potential N-glycosylation sites were mutated by conversion of the corresponding Asn residues to Gln to assess the role of these N-glycosylation sites in the activity, stability, and expression of rPAE.

2. Materials and methods

2.1. Site-directed mutagenesis

Three potential N-glycosylation sites (N43, N212, and N280) in the rPAE amino sequence were mutated by replacing the Asn (AAC) residues with Gln (CAA). Mutagenic primers (synthesized by Shanghai Generay Biotech Co., Ltd., China) are listed in Table 1. The desired mutations were introduced by PCR; the reactions were performed using 1.5 μl of each primer (10 μM) for 15 cycles of 30 s at 95 °C, 1 min at 55 °C, and 6 min at 68 °C. For constructing single mutants, PCR reactions were performed using pGH-T plasmid (Shanghai Generay Biotech Co., Ltd., China) inserted with wild-type *las*B as the template. The resulting amplified products were subjected to *Dpn*I digestion to remove the parental DNA template, and subsequently transformed into *E. coli* DH5α for mutant plasmid amplification. All mutants were subjected to DNA sequencing analyses to verify the desired mutations. The mutated *las*B genes were released from the pGH-T plasmids by digestion with *Eco*RI and *Avr*II, and then sub-cloned in pPIC9K (Invitrogen, Carlsbad, CA, USA). Double and triple mutants were constructed by the sequential accumulation of mutations.

Table 1
Mutagenic primers.

Mutations	Mutagenic primers*
N43Q	5′-CATCACCGTCGACATGCAAAGCAGCACCGACGAC-3′ (sense)
	5′-GTCGTCGGTGCTGCTTTGCATGTCGACGGTGATG-3′ (anti-sense)
N212Q	5′-GGGCGATCCATCGACCAAGCGTCGCAGTACTAC-3′ (sense)
	5′-GTAGTACTGCGACGCTTGGTCGATGGATCGCCC-3′ (anti-sense)
N280Q	5′-GCGCAGAACCGCCAATACTCGGCGGCTGAC-3′ (sense)
	5′-GTCAGCCGCCGAGTATTGGCGGTTCTGCGC-3′ (anti-sense)

* Underlines indicate the mutated sites.

2.2. Transformation and expression

The recombinant pPIC9K (Invitrogen) harboring the gene of interest was linearized by *Sac*I (Fermentas, Canada) and then, using Gene Pulser (Bio-Rad, Hercules, CA, USA), electroporated into competent cells of *P. pastoris* KM71 (Invitrogen) prepared according to the manufacturer's instructions. The transformed cells were plated on minimal dextrose (MD) agar and cultured at 28 °C for 4 days. The transformants were picked and then cultured in 25 ml of BMGY medium at 28 °C for 24 h. Cells were centrifuged, resuspended in approximately 10 ml BMMY medium until a final OD600 of approximately 40 was reached. The cells were then incubated with shaking at 250 rpm at 22 °C for 5 days with 1% methanol added to the medium daily.

2.3. Purification of non-glycosylated and glycosylated rPAE

The non-glycosylated (the 33-kDa protein expressed by the N43Q/N280Q strain) and glycosylated rPAE proteins (the 35- and 37-kDa forms expressed by the wild-type strain) were purified. The culture supernatant of the corresponding strains was concentrated using ultrafiltration spin columns (Sartorius, Göttingen, Germany). The resultant sample was applied to a DEAE Sepharose Fast-Flow column and eluted with a linear gradient of 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing NaCl from 0 to 0.5 M. The fractions with caseinolytic activity were pooled and concentrated. The resulting sample was loaded onto a Sephadex G-75 gel-filtration column and eluted with 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). The proteins from the column were analyzed by SDS-PAGE, and the desired rPAE glycoforms were obtained from the corresponding fractions.

2.4. Caseinolytic activity assay

A 50 μl-aliquot of the enzyme solution was added to 2.0 ml of 1% (w/v) casein (Sigma, St. Louis, MO, USA) solution containing 50 mM barbital/HCl (pH 7.0) and incubated at 60 °C for 30 min. The reaction was terminated by adding 2.0 ml of 10% (w/v) trichloroacetic acid. The mixture was incubated at 37 °C for 20 min and then centrifuged at 10,000 × *g* for 10 min. One milliliter of the resulting supernatant was added to 5 ml of 2% (w/v) Na_2CO_3 containing 0.4% (w/v) NaOH and 1 ml Folin–Ciocalteau reagent, and the mixture was then incubated at 40 °C for 15 min. The sample absorbance was read at 750 nm. One caseinolytic activity unit (U) was defined as the amount of enzyme that produces an absorbance equivalent to 1 μg tyrosine liberated per minute under the test conditions.

2.5. Protein quantitation assay

Protein concentrations were determined at 750 nm using the Lowry method [21], with bovine serum albumin (BSA) as the standard.

2.6. Peptide synthesis reactions

The reactions were performed in 50% (v/v) DMSO and 50 mM sodium–phosphate buffer (pH 7.0) at 30 °C. The reaction mixture was incubated at 30 °C for 2 days for determination of the peptide synthesis yield and for one hour for determination of the peptide synthesis rate. The substrate concentrations were 50 mM for Cbz-Ala, Cbz-Asp, Cbz-Thr, and Cbz-Arg and 100 mM for Phe-NH_2 and Leu-NH_2. The peptide synthesis substrates were obtained from Shanghai Hanhong Chemical Co., Ltd., China. All enzymes had a concentration of 0.021 mg/ml.