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Modification of recombinant elastase expressed in *Pichia pastoris* by introduction of N-glycosylation sites

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ABSTRACT

A novel N-glycosylation site was introduced into recombinant elastase (rPAE) at N36, N67, or N264 through the site-directed mutagenesis of I38T, A69T, or N266T, respectively. The A69T mutation completely inhibited the expression of rPAE. As expected, the I38T and N266T mutant proteins exhibited higher degrees of N-glycosylation compared with the wild type rPAE. The I38T mutant was more efficient in the hydrolysis of casein in aqueous medium and exhibited higher specific activity and k_{cat} values and a lower K_m value. In contrast, the N266T mutant and the wild type displayed similar values. Importantly, the I38T mutant achieved in higher rates and yields of peptide synthesis in 50% (v/v) dimethylsulfoxide, whereas the N266T mutant was similar to the wild type rPAE. Furthermore, the maximum yield of Z-Ala-Phe-NH2 synthesis catalyzed by the I38T mutant protein (87%) was higher than those achieved by the wild type (78%) and N266T mutant (78%) proteins. Neither the I38T nor the N266T mutation exerted significant effects on the rPAE solvent stability. In aqueous medium, the I38T mutation decreased the rPAE thermostability, and the N266T mutation slightly improved that. In conclusion, the I38T mutation improved the potential of rPAE in industrial applications.

1. Introduction

Recently, significant attention has been paid to proteases that are stable and active in organic solvents, and there are many advantages to the application of these enzymes in peptide synthesis, such as their high regioselectivity and stereoselectivity and their minimal side-chain protection requirements (Doukyu and Ogino, 2010). Among these proteases, the *Pseudomonas aeruginosa* elastase (PAE) is one of the most promising catalysts in this field. It has excellent stability and high enzymatic activity in organic solvents, which results in high equilibrium yields for peptide synthesis (Doukyu and Ogino, 2010; Tang et al., 2010; Tsuchiyama et al., 2007; Ogino et al., 1999a, 1999b, 2000). PAE is encoded by the *las*B gene and is also called pseudolysin (EC 3.4.24.26) (Bever and Iglewski, 1988). It is a zinc metalloprotease composed of 301 amino acid residues. Its molecular weight is 33 kDa, and it has a pI of 5.9. Its optimum pH and temperature are 7.5–8.0 and 60 °C, respectively (Cheng et al., 2009; Han et al., 2012; Ogino et al., 1999a).

In contrast, *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen, and the fermentation of *P. aeruginosa* produces some toxic metabolites (Döring and Pier, 2008). To facilitate the industrial application of this enzyme, our lab cloned the *las*B gene (GenBank Accession No. JX003619), inserted it into pPIC9K, and heterologously expressed the gene in a safe and efficient host, i.e., *Pichia pastoris* KM71. The recombinant elastase (rPAE) was secreted outside the cells and heterogeneously N-glycosylated (Han et al., 2013).

N-glycosylation is one of the most common forms of protein post-translational modifications in *P. pastoris*. Furthermore, N-glycosylation often significantly affects the secretion, stability, and activity of heterologous proteins (Damasceno et al., 2012; Skropeta, 2009). The objectives of this study were to modify rPAE protein by introduction of novel N-glycosylation sites to it: the effect of the additional N-glycosylation of rPAE at N36, N67, or N264 on its stability and activity was evaluated.

2. Methods

2.1. Site-directed mutagenesis

Three novel N-glycosylation sites were introduced through site-directed mutagenesis into rPAE at N36, N69, and N264 by the

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Table 1
Mutagenic primers.

Mutations	Mutagenic primers
I38T	5′-CGACGGCAACGTCACCACCGTCGACATGA-3′ (forward)
	5′-TCATGTCGACGGTGGTGACGTTGCCGTCG-3′ (reverse)
A69T	5′-AAGCAGGTCAACGGCACCTATTCGCCGCTGA-3′ (forward)
	5′-TCAGCGGCGAATAGGTGCCGTTGACCTGCTT-3′ (reverse)
N266T	5′-CACCAGCAACTACACCAGCGGCGCCTG-3′ (forward)
	5′-CAGGCGCCGCTGGTGTAGTTGCTGGTG-3′ (reverse)

The underlined letters indicate the mutated sites.

mutations I38T, A69T, and N266T. The 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. To construct the single mutants, PCR reactions were performed using the pGH-T plasmid (Shanghai Generay Biotech Co., Ltd., China) 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 cells for mutant plasmid amplification. All of the mutants were subjected to DNA sequencing analyses to verify the desired mutations. The mutagenic *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). The double and triple mutants were constructed by the sequential accumulation of mutations.

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), which were prepared according to the manufacturer's instructions. The transformed cells were plated on minimal dextrose (MD) agar and cultured at 28 °C for four days. The transformants were picked and then cultured in 25 ml of BMGY medium at 28 °C for 24 h. The cells were centrifuged and resuspended in approximately 10 ml of BMMY medium; the final OD600 was adjusted to approximately 40. The cells were then incubated at 250 rpm and 22 °C with shaking for five days, and 1% methanol was added daily to the medium.

2.3. Purification of mutant rPAE

The I38T and N266T mutant rPAE proteins and the wild type protein were purified before their stabilities and activities were assayed. 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 equilibrated with 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). Elution was performed 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 subjected to a caseinolytic activity assay, and the desired proteins were obtained from the corresponding fractions.

2.4. Caseinolytic activity assay

A 50 μl-aliquot of the enzyme solution obtained through the above-described procedure 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 the mixture was incubated at 60 °C for 30 min. The reaction was terminated through the addition of 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 of Folin–Ciocalteu reagent, and the mixture was 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 of tyrosine liberated per minute under the test conditions.

2.5. Protein quantitation assay

The protein quantification was performed at 750 nm according to the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard.

2.6. Protein stability in aqueous and organic solvents

The concentration of each enzyme was 200 U/ml. The residual enzymatic activities in 50 mM sodium-phosphate buffer (pH 7.0) were determined after incubation at 70 °C. The enzymatic activities prior to heat treatment were set to 100%. As for assay of their solvent-stabilities, the purified enzymes in 50 mM sodium-phosphate buffer (pH 7.0) and 50% (v/v) organic solvents were incubated at 30 °C for 4 days with constant shaking at 140 rpm, then their residual activities were determined.

2.7. Peptide synthesis reactions

The reactions were performed in 50% (v/v) dimethylsulfoxide and 50 mM sodium-phosphate buffer (pH 7.0) at 30 °C. Each enzyme was purified and added at a concentration of 0.035 mg/ml whose specific activity was presented in Table 2. The reaction mixture was incubated at 30 °C for two days to determine the peptide synthesis yield and for one hour to determine the peptide synthesis rate. The substrate concentration of Cbz-Ala, Cbz-Asp, Cbz-Thr, and Cbz-Arg was 50 mM, and the substrate concentration for Phe-NH_2 and Leu-NH_2 was 100 mM. The peptide synthesis substrates were obtained from Shanghai Hanhong Chemical Co., Ltd. (China).

Table 2
Michaelis–Menten kinetic parameters of caseinolytic activity.

Enzyme	Specific activity U/mg	K_m gl^{-1}	k_{cat} s^{-1}	k_{cat}/K_m lg^{-1} s^{-1}
Wild type	2427 ± 79	2.77 ± 0.33	0.0512 ± 0.0012	0.018484 ± 0.00177
I38T	2621 ± 123	2.39 ± 0.22	0.0587 ± 0.0029	0.024561 ± 0.00105
N266T	2417 ± 131	2.71 ± 0.43	0.0518 ± 0.0021	0.019114 ± 0.00226

Reactions were performed in 40 mM barbital/HCl buffer (pH 7.5) at 60 °C for 30 min using casein (1.25, 2.5, 5, and 10 g/l) as the substrate in the presence of each purified enzyme. The kinetic parameters values were calculated from a nonlinear regression fit to the Michaelis–Menten equation. Each value represents the average and standard deviation ($n = 3$).