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N-糖基化对毕赤酵母表达的 DSPAα1 分泌和活性的影响

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摘　要: 溶栓剂 DSPAα1 正处于治疗急性缺血性中风的 III 期临床研究，临床结果显示 DSPAα1 具有良好的药理学和安全特性。将 DSPAα1 基因序列按照毕赤酵母偏好密码子进行优化，并在毕赤酵母菌株 GS115 和 KM71 中进行表达，同时利用定点突变对糖基化侧链进行缺失，考察糖基侧链对毕赤酵母表达 DSPAα1 的影响。结果表明，野生型 DSPAα1 在 GS115 和 KM71 中均获得高表达，在摇瓶发酵条件下，表达量分别为 70 mg/L 和 105 mg/L; 利用 SDS-PAGE 对 DSPAα1 三种突变体 (N117Q、N362Q 和 N117Q/N362Q) 进行分析，与野生型蛋白质相比较，3 种突变体的表达水平显著下降，同时纤溶平板测活数据显示，纯化后的突变体 N117Q 和 N362Q 比活性均低于野生型蛋白质的 25%。这表明，N-型糖链 (N117 和 N362) 对毕赤酵母表达的 DSPAα1 分泌和酶活性具有重要作用。

关键词: N-糖基化，重组 DSPAα1，毕赤酵母

Effect of N-linked glycosylation on secretion and activity of recombinant DSPAα1 expressed in *Pichia pastoris*

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Abstract: The thrombolytic agent DSPAα1 is currently undergoing clinical trials for the treatment of acute ischemic stroke and has shown good pharmacodynamic, pharmacokinetic and safety profiles. Here, the DSPAα1 gene, optimized for the preferred codons of yeast, was cloned into the *Pichia pastoris* strains GS115 and KM71. Both expression systems produced functional DSPAα1 into the broth medium under shaking flask growth conditions with the yield of about 70 mg/L, and 105 mg/L, respectively. In addition, three glycosylation minus DSPAα1 mutants, constructed by site-directed mutagenesis, were also expressed in *Pichia pastoris*. The mutant proteins were assayed by SDS-PAGE and fibrin degradation activities were evaluated. The secretion levels of all the mutants, especially N362Q and N117Q/N362Q, were so lower compared to the wild-type DSPAα1 that only minimal quantities of mutant protein could be recovered by purification from the culture medium. The protein specific activities from mutants (N117Q, N362Q) were less 25% than that of the wild type protein. These results imply that the N-linked carbohydrate chains (at N117 and N362) are vital for the enzymatic activity of rDSPAα1 and for its secretion from *Pichia pastoris*.

Keywords: N-linked glycosylation, recombinant DSPAα1, *Pichia pastoris*

Introduction

DSPAs (*Desmodus rotundus* salivary plasminogen activators) are a group of serine proteases with plasminogen activator (PA) activity, and are comprised of four distinct variants: α1, α2, β and γ [1]. Compared

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to the currently commonly available PAs, DSPA proteins show remarkably effective thrombolytic activity, especially in the case of DSPAα1. DSPAα1 consists of several distinct peptide regions: a finger domain, an epidermal growth factor region, a kringle domain and a serine protease domain[1-2]. The peptide backbone of DSPAα1 includes two N-glycosylation sites at residues Asn-117 and Asn-362, as predicted by the consensus sequence Asn-X-Ser/Thr[3]. In contrast to tPA (tissue-type plasminogen activator), DSPAα1 has exceptionally high fibrin specificity, and in animal models has shown remarkable efficacy for thrombolysis, low incidence of systemic bleeding, low reocclusion rate, an effective time window extending up to nine hours, and the absence of potential neurotoxic effects[4-7]. These superior properties imply that DSPAα1 presents a potentially safer and more efficacious thrombolytic agent than tPA. The clinical trials of DSPAα1 in acute ischemic stroke studies also demonstrated that it is a promising thrombolytic agent[8].

Originally, natural DSPAα1 was purified directly from vampire bat salivary gland, which clearly limited its wider application. Subsequently, recombinant DSPAα1 has been produced in a number of different host cell systems, including baby hamster kidney (BHK)[9], Chinese hamster ovary (CHO)[10], insect (Sf 9)[10] and plant (tobacco)[11]. *Pichia pastoris*, a methylotropic yeast, has been developed as a host for heterologous protein expression because of its low costs, high expression levels, efficient secretion, post-translation modification, proper protein folding and the potential for very high cell density fermentation[12-14]. However, some groups have reported that a few proteins expressed in yeast contain non-human classes of N-linked glycans of the high-mannose type, which can be immunogenic in humans and can also lead to accelerated clearance, thus limiting their therapeutic value[15-16]. The direct solution of this problem is to eliminate the N-linked glycan in the synthesized protein through appropriate site-directed mutagenesis of the coding gene, but sometimes such mutations can result in major effects on the folding, activity and secretion of the enzyme [17-20].

In this study, an efficient and cost-effective *Pichia pastoris* expression system was developed to produce recombinant DSPAα1, and the two glycosylation sites (Asn-117 and Asn-362) were eliminated by site-specific mutation to study the influence of the N-linked glycans on protein expression and enzyme activity.

1 Materials and methods

1.1 Strains, reagents and media

The *Pichia pastoris* strains GS115, KM71 and the expression vectors pPIC9, pPIC9K were obtained from Invitrogen (California, USA). All enzymes for DNA applications were purchased from Promega. The media used, buffered minimal methanol (BMMY), buffered minimal glycerol (BMGY) and minimal dextrose (MD), were prepared following the manual for the *Pichia pastoris* expression kit (Invitrogen). The GeneEditor™ *in vitro* Site-Directed Mutagenesis System kit was purchased from Promega.

1.2 Synthesis of DSPAα1 and site-directed mutagenesis to produce non-glycosylated DSPAα1

The codon-optimized DNA sequence of DSPAα1 was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (China), and contained *Xho* I and *Eco*R I sites at the 5′ and 3′ termini, respectively. In addition, the 5′ terminus also contained a site for *kex2* cleavage (GenBank accession GQ412732).

The GeneEditor™ *in vitro* Site-Directed Mutagenesis System was used to create the Asn117 and Asn362 to Gln mutations, individually or together. The mutant primers included pM117: 5′-pGTATCAACTGG CAGAGCAGCCTTCTG-3′ and pM362: 5′-pCAAGT TTCTGTTTCAGAAAACCGTC-3′. Briefly, the oligonucleotide primers (representing the mRNA strand), containing the desired mutation (Asn117 to Gln117, or Asn362 to Gln362), were annealed to the synthesized DSPAα1 DNA and extended by temperature cycling using the very high fidelity enzyme T4 DNA polymerase, then ligated by using T4 DNA ligase. The mutant plasmids were transformed into the *E. coli* strain BMH71-18 and positive transformants were cultured in LB broth containing GeneEditor™ Antibiotic Selection Mix. The plasmids were purified and transformed into JM109 competent cells, and clones were selected and their DNA sequences were determined to confirm the desired mutation.

1.3 Construction of expression plasmids

The wild-type DSPAα1, or the three mutant genes, were digested with *Xho* I and *Eco*R I, and then inserted into the vector pPIC9 cut at the *Xho* I and *Eco*R I sites, to yield plasmids pP9DSPAα1 (s). Then the four genes were cloned into pPIC9K to yield pP9KDSPAα1 (s). The plasmid pPIC9K contains an AOX1 promoter that allows methanol-inducible high-level expression in *Pichia pastoris*, an α-factor signal sequence for secretion, and a kanamycin resistance gene and a

histidinol dehydrogenase gene (*his4*) for positive clone selection.

1.4 Construction and selection of recombinant yeast

The four recombinant plasmids pP9KDSPAα1 (s), linearized with *Sal* I, were transformed into competent *Pichia pastoris* cells by electroporation. 1 mL of ice-cold 1 mol/L sorbitol was added to the electroporation cuvette immediately after pulsing. His^+ transformants were selected on histidine-deficient MD agar plates. Several His^+ clones were randomly picked and inoculated into YPD (1% yeast extract, 2% tryptone, 2% dextrose). The transformants were verified by PCR using gene-specific primers.

1.5 Expression of recombinant yeast

Several recombinant clones from YPD were grown in 20 mL of BMGY medium in 100 mL Erlenmeyer flasks and incubated at 30°C with shaking at 200 r/min until the OD_{600} was 10−20. Cells were collected by centrifugation at 4 000 r/min for 5 min and resuspended in BMMY medium (pH 6.0), then transferred to 50 mL BMMY media in 500 mL Erlenmeyer flasks. The final OD_{600} was approximately 2.0. The cultures were grown at 20°C with shaking at 250 r/min for a week. Methanol was added once a day at a concentration of 1:200 (*V*/*V*).

1.6 SDS-PAGE and fibrin degradation assay of DSPAα1

The culture supernatants were collected by centrifugation at 4 000 r/min for 10 min. The supernatant and cell lysate were analyzed by reducing sodium dodecyl sulfate (SDS), 12% polyacrylamide gel electrophoresis (PAGE). The concentration of recombinant DSPAα1 protein was assayed by SDS-PAGE based on a standard curve generated by the purified recombinant DSPAα1, which was quantified by the Bradford protein quantitation method. Enzyme activities were detected by a modified fibrin assay as described[21]. A fibrin-agarose gel plate was prepared by mixing 0.125 mg agarose in 25 mL saline, adding 13.2 mg fibrinogen, 14 μL 100 IU/mL of thrombin and 280 μL 0.5 mg/mL plasminogen. About 10 μL of supernatant from cultures of transformants was applied to each well on the plate and incubated at 37°C for six hours.

2 Results

2.1 Heterologous expression of DSPAα1 (wild-type) in *Pichia pastoris*

The DSPAα1 gene DNA sequence, which incorporated yeast-preferred codons, was 1 434 bp in length, encoding a 477 amino acid mature protein. Insertion of the cDNA sequence into the vector pPIC9K between the *Xho* I and *Eco*R I sites produced a 10.8 kb plasmid. Since the constructed plasmid contained an α-factor signal peptide sequence in front of the DSPAα1 gene, the expressed recombinant DSPAα1 was secreted out of the host cells.

The plasmids pP9KDSPAα1 (wild-type) and pPIC9K were linearized with *Sal* I and transformed into the *Pichia pastoris* cells GS115 and KM71. Selection on MD plates yielded thousands of His^+ transformants. Thirty colonies from GS115 and KM71, respectively, plus the control strain, were randomly picked into YPD medium, and transferred to BMMY for further evaluation in submerged fermentation analysis. The transformants' broth supernatants were analyzed by SDS-PAGE where a protein band (62 kDa) was clearly observed and absent in the control strain (Fig. 1A). Little difference in DSPAα1 expression levels was observed among the GS115 or the KM71 cultures (data not shown), although the DSPAα1 expression levels for KM71 were about 1.5 times more than for GS115 (70 mg/L), up to 105 mg/L (Fig. 1B). Three colonies from the thirty KM71 or GS115 tested, named KM71-DSPAα1-1~3 and GS115-DSPAα1-1~3, respectively, were further analyzed in detail.

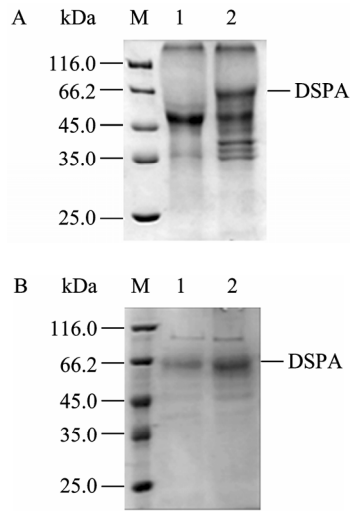

Fig. 1 SDS-PAGE analysis of the recombinant protein DSPAα1. (A) M: protein marker; 1: concentrated supernatant from the negative control after cultured 5 days; 2: concentrated broth supernatant from the GS115-DSPAα1-1 after cultured 5 days. (B) M: protein marker; 1: supernatant from the GS115-DSPAα1-1 from a 5-day culture; 2: supernatant from KM71-DSPAα1-1 from a 5-day culture.

Since the recombinant DSPAα1 was able to cleave plasminogen into plasmin to degrade fibrin, the PA (plasminogen activator) activity of the recombinant protein in transformants' broth supernatant was analyzed by the fibrin assay. The expression levels of three positive transformants (from GS115 and KM71) were monitored and showed that the protein expression levels continued to increase from day 1 to day 7 following methanol induction (Fig. 2). By day 8, the pH of the supernatant had increased and the recombinant DSPAα1 yield began to decrease.

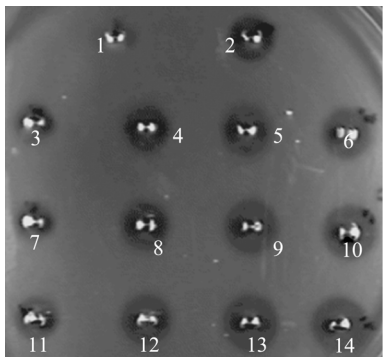

Fig. 2 Fibrin assay of recombinant DSPAα1 in broth supernatant of transformants. 1: the negative control; 2: the positive control tPA; 3-6: GS115-DSPAα1-1 (24 h, 72 h, 120 h, 168 h); 7-10: GS115-DSPAα1-2 (24 h, 72 h, 120 h, 168 h); 11-14: GS115-DSPAα1-3 (24 h, 72 h, 120 h, 168 h).

2.2 Analysis of the DSPAα1 mutants

The DSPAα1 mutant transformants were selected in the same way as the wild-type DSPAα1. The expression from *Pichia pastoris* of glycosylated DSPAα1 and the DSPAα1 non-glycosylated at one or two sites (Fig. 3A) was studied and the PA activity of the DSPAα1 mutants was also evaluated. The three DSPAα1 mutant transformants were assayed by flask fermentation. As shown in Fig. 3B, the apparent molecular weight of the DSPAα1 mutant N117Q in culture was decreased to 58 kDa comparing to the wild-type DSPAα1, which was taken as proof that the sugar chain of Asn117 had been eliminated. The results of N362Q and N117Q/N362Q showed that the expression levels for these two mutants were dramatically decreased such that they couldn't be detected by SDS-PAGE. However, compared to wild DSPAα1, the cell lysate of three mutants all had an extra concentrated protein band with an apparent size of 70 kDa corresponding to DSPAα1 with a signal peptide at the N terminus. The enzyme activites were also detected, and the results showed that the mutants enzyme activites dramatically decreased (Fig. 3C).

After purification and detection, the specific activity of the mutants N117Q and N362Q was less than 25% of the wild-type DSPAα1. The yield for the third mutant N117Q/N362Q was so low that enough protein couldn't be obtained to assay.

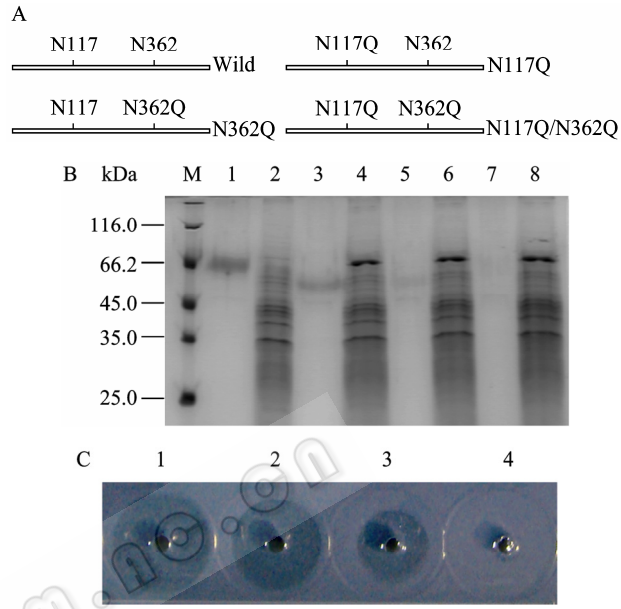

Fig. 3 Analysis of the DSPAα1 mutants. (A) Schematic representation of N-glycosylation mutants. (B) SDS-PAGE analysis of the recombinant protein DSPAα1 and mutants. M: protein marker; 1: supernatant from the wild DSPAα1; 2: cell lysate of the wild DSPAα1; 3: supernatant from the DSPAα1 mutant N117Q; 4: cell lysate of the DSPAα1 mutant N117Q; 5: supernatant from the DSPAα1 mutant N362Q; 6: cell lysate of the DSPAα1 mutant N362Q; 7: supernatant from the DSPAα1 mutant N117Q/N362Q; 8: cell lysate of the DSPAα1 mutant N117Q/N362Q. (C) Fibrin assay of DSPAα1 mutants in broth supernatant of transformants. 1: wild DSPAα1; 2: DSPAα1 mutant N117Q; 3: DSPAα1 mutant N362Q; 4: DSPAα1 mutant N117Q/N362Q.

3 Discussion

It has been reported in the literature that DSPAα1 has been expressed in various hosts, namely insect, tobacco and mammalian cells. The expression in insect and tobacco cells is problematic because of low expression levels and, for tobacco, also high levels of proteolysis. On the other hand, the mammalian cell expression systems are high in cost and require stringent control procedures to detect possible infectious agents. The ability of the yeast *Pichia pastoris* to be grown rapidly on simple media to high cell density, with secretion of expressed foreign proteins gives it advantages over other expression systems[22]. Therefore, in this study *Pichia pastoris*

was chosen to express DSPAα1. To our knowledge, this is the first time that DSPAα1 has been expressed in *Pichia pastoris*.

Pichia pastoris is a widely used industrial methylotrophic yeast which has been developed as a host for expression of foreign proteins[23]. In this study, the results indicated that functional DSPAα1 was successfully expressed in two *Pichia pastoris* strains and the yield of the recombinant DSPAα1 in KM71 was up to 105 mg/L, which is higher than those obtained in other expression systems (the highest reported yield in CHO cells is 60 mg/L)[9-11]. The *Pichia pastoris* strain KM71, with its slow methanol utilization (MutS) genotype could be predicted to be better for high-level expression of DSPAα1 than is GS115 which has wild-type methanol utilization (Mut+) genotypic expression system. These well known effects are related to the *AOX* promoter and its repression. In the case of DSPAα1, the 14 disulfide bonds of this protein may require extended times to permit proper post-translational processing[1]. Our results do show that *Pichia pastoris* is the most efficient host among those systems reported to express functional DSPAα1. Given the good yields of recombinant DSPAα1 produced under the shaking flask conditions, the prospects look promising for optimizing this expression system to produce recombinant DSPAα1 on a large scale[24]. In our experiments the expressed recombinant DSPAα1 produced by the two strains was the dominant protein found in the medium supernatant which should simplify purification of the protein in industrial settings.

As shown in SDS-PAGE, the recombinant DSPAα1 produced in *Pichia pastoris* is a hyper-glycoenzyme (apparent size 62 kDa), which contains about 24% carbohydrate content and these glycan maybe limited DSPAα1 therapeutic value[20]. To attempt to overcome these drawbacks, three glycosylation minus DSPAα1 mutants were constructed to evaluate the role of the sugar chains in the DSPAα1 protein. The results showed that the apparent molecular weights of mutants were clearly decreased. Although the intracellular protein concentrations were essentially normal, the greatly reduced or largely absent quantities of protein recoverable from the broth supernatant strongly suggests that the secretion of the non-glycosylated proteins was impaired as a result of the glycan deletion. The extracellular protein levels for mutant N362Q was less than for the mutant N117Q, thus implying that the glycan at Asn362 is more important for secretion of DSPAα1 than that at Asn117. The double mutant N117Q/N362Q could not be detected in the broth supernatant because of the very low yield. These results indicated that both glycosylation sites (Asn117 and Asn362) played important roles in the secretion of DSPAα1 from *Pichia pastoris*. The specific activities of each were also assayed and both mutants (N117Q and N362Q) showed less than 25% of the activity of wild-type DSPAα1. This demonstrated that the N-linked glycans of the secreted DSPAα1 are also critical for DSPAα1's enzyme activity which has also been reported in other studies[19,25].

Unfortunately, these study results are discouraging because the deletion of the two N-glycans had a dramatic influence on both the secretion and the enzymatic activity of DSPAα1. This demonstrated that recombinant, non-glycosylated DSPAα1 is of no practical use. However, there have been encouraging recent advances involving the glycoengineering of *Pichia pastoris* that have resulted in the humanizing of the protein glycosylation pathways in this cell[26-27]. Glycosylated recombinant proteins synthesized in these humanized yeast cells can be predicted to show better overall characteristics because of their native glycosylation properties. Improving the specific activity of non-glycosylated DSPAα1 through protein engineering is also a realistic and practical approach. These alternative solutions will be pursued in the future.

In conclusion, we report here that DSPAα1 has been expressed successfully in *Pichia pastoris*. The expression levels of DSPAα1 could be enhanced further by optimizing culture fermentation conditions or by enlarging the scale of culture. N-glycosylation of this protein is critical in order to maintain enzyme activity and for efficient secretion of the DSPAα1 protein from *Pichia pastoris*.

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