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Degradation of HSA-AX15(R13K) when expressed in *Pichia pastoris* can be reduced via the disruption of YPS1 gene in this yeast

Xue Qin Yao, Hong Liang Zhao, Chong Xue, Wei Zhang, Xiang Hua Xiong, Zhi Wei Wang, Xian Yu Li, Zhi Min Liu*

Department of Microorganism Engineering, Beijing Institute of Biotechnology, 20 Dongdajie Street, Fengtai District, Beijing 100071, People's Republic of China

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ABSTRACT

Expression of recombinant protein HSA-AX15(R13K) in *Pichia pastoris* GS115 strain produced both the intact protein and its two degradation products with molecular weights of around 43 kDa and 66.2 kDa, respectively. To reduce or avoid the degradation, a modified *P. pastoris* GS115 stain, in which YPS1 gene was disrupted, was constructed via homologous recombination and used as a host strain for the HSA-AX15(R13K) expression. After 60 h of induction during culture, it was found that the degradation product of around 66.2 kDa was reduced significantly in the supernatant of yps1-disrupted strain compared with that in the supernatant of wild-type strain. By the Western blot analysis of culture supernatants from wild-type and yps1-disrupted strains expressing HSA-AX15(R13K), the significant improvement was also seen in the degradation product of around 43 kDa. Comparison of cell growth between the two strains demonstrated a similar growth tendency, thereby indicating that the disruption of YPS1 gene has no effect on the normal physiology of GS115 strain. Following induction for 60 h, the yield of intact HSA-AX15(R13K) in the yps1 disruptant was three-fold higher than that in the wild-type strain. Therefore, such a *P. pastoris* mutant deficient in YPS1 activity is suitable for the high-level expression of recombinant protein HSA-AX15(R13K).

1. Introduction

Ciliary neurotrophic factor (CNTF), synthesized in glial cells within the central and peripheral nervous systems, had been used clinically as a natural leptin analogue to treat obesity (Sleeman et al., 2000). A recombinant human CNTF mutant, known as AX15, was constructed by replacing free cysteine at position 17 and free glutamine at position 63 in the amino acid sequence of wild-type CNTF with alanine and arginine, respectively, and by removing last 15 amino acid residues (Lambert et al., 2001; Graewin et al., 2006). But the degradation of AX15 when expressed in *Pichia patoris* occurred. Because of this, an AX15 mutant, named AX15(R13K), was constructed by the replacement of arginine at position 13 with lysine, and as a result, an intact protein was produced without any clearly degraded products (Zhao et al., 2004). To prolong the *in vivo* half-life of AX15(R13K), a recombinant protein HSA-AX15(R13K) was constructed via fusion of AX15(R13K) to C-terminal of HSA via an linker containing 11 amino acids (Zhao et al., 2005).

Unexpectedly, the degradation of HSA-AX15(R13K) when expressed in *Pichia pastoris* GS115 strain was also found with two degradation products with molecular weights of around 43 kDa and 66.2 kDa, respectively. Similarly, when *Saccharomyces cerevisiae* or *P. pastoris* was used to produce recombinant human serum albumin (rHSA), there was a minor N-terminal fragment of around 43 kDa except for major 67 kDa mature albumin in supernatant (Kerry-williams et al., 1998; Kobayashi et al., 2000). Researchers had reported that this degradation phenomenon occurring in *S. cerevisiae* was related to the members of Yapsin family, particularly Yapsin 1, which is encoded by YPS1 gene in *S. cerevisiae*, is an aspartic protease with an optimum PH of 4.0–5.0, and can recognize not only paired basic residues but also a monobasic residue accompanied by an additional basic amino acid at a specific upstream or downstream position (Azaryan et al., 1993; Ash et al., 1995; Ledgerwood et al., 1996; Cawley et al., 1996). Moreover, cleavage catalyzed by yps1 endoprotease occurs on carboxyl side of paired basic residues or between them (Azaryan et al., 1993), and only on carboxyl side of monobasic residue (Ledgerwood et al., 1996). It is well established that YPS1 is a glycophosphatidylinositiol-anchored protein that localizes to cell membrane and partially to other different subcellular locations such as late Golgi apparatus and vacuole (Ash et al., 1995; Sievi et al., 2001). In addition, Werten and Wolf (2005) had cloned and characterized the YPS1 gene of *P. pastoris*. According to the above, it is supposed that the YPS1 gene-encoded product in *P. pastoris* may be responsible for the

* Corresponding author. Tel.: +86 10 66948823; fax: +86 10 63833524.
E-mail address: liuzhm@vip.sina.com (Z.M. Liu).

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degradation of HSA-AX15(R13K). To reduce the degradation caused by the YPS1 gene-encoded product, many methods are available including optimization of medium by adding arginine or ammonium salt to prevent acidification of medium PH during cultivation, mutagenesis of yps1 endoprotease recognition site, addition of pepstatin A to inhibit activity of yps1 endoprotease, and disruption of YPS1 gene via homologous recombination (Kerry-williams et al., 1998; Kang et al., 1998, 2000). However, it is well known that gene disruption is a more effective method for gene inactivation compared with those other than the disruption-based method. For instance, many yps1 disruptants were constructed in *Pichia angusta* (previously named *Hansenula polymorpha*) and *S. cerevisiae*, and then obviously reduced the decomposition of heterologous proteins, e.g. human pre-elafin, hHSA, hHSA-hGH, hPTH and HSA-TIMP2 (Kerry-williams et al., 1998; Bourbonnais et al., 2000; Kang et al., 2004). Therefore, these above-mentioned studies suggest that reduction of HSA-AX15(R13K) degradation via the disruption of *P. pastoris* YPS1 gene may also be feasible.

Werten and Wolf (2005) reported a yps1-disrupted *P. pastoris* strain which, however, did not achieve their expectation due to not reducing degradation of gelatin at all. By contrast, in this study the significant reduction of HSA-AX15(R13K) degradation was achieved by the yps1-disrupted GS115 strain which was constructed via homologous recombination between YPS1 gene and YPS1 mutant gene created *in vitro*. Many experimental data will be provided in this text to support this new finding. The reduced degradation of HSA-AX15(R13K) during its expression in *P. pastoris* demonstrates that such a yps1-disrupted strain is a suitable host for the high-level of production of HSA-AX15(R13K).

2. Materials and methods

2.1. Plasmid, strains and culture media

The plasmid pPIC9-HSA-AX15(R13K) was used for expression of HSA-AX15(R13K) in *P. pastoris*, as described previously (Zhao et al., 2005). *P. pastoris* GS115 strain (Invitrogen), defective in histidine dehydrogenase gene (*HIS4*), was used as a host strain for expression of HSA-AX15(R13K). *Escherichia coli* DH5α was used for cloning purpose.

Yeast extract peptone dextrose (YPD) medium composed of 10 g l^{-1} yeast extract (Oxoid), 20 g l^{-1} peptone (Oxoid) and 20 g l^{-1} glucose (Merck) was used for culturing all *P. pastoris* strains. Minimal dextrose (MD) medium consisting of 13.4 g l^{-1} yeast nitrogen base (YNB) without amino acids (Difco), 20 g l^{-1} glucose and 0.4 mg l^{-1} biotin (Roche) was used to select *His*$^+$ transformants. YPDS (*Zeocin* plus) medium containing 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose, 1 M sorbitol (Amresco) and 100 mg l^{-1} *Zeocin* (Invitrogen) was used for the selection of *Zeocin*$^+$ transformants. For induction of HSA-AX15(R13K) fusion gene, *His*/*Zeocin*$^+$ transformants were grown at 30 °C in 250 ml shake-flasks containing buffered minimal glycerol or methanol (BMGY/BMMY) medium, which consists of 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g l^{-1} YNB, 0.4 mg l^{-1} biotin, and either 1% (v/v) glycerol (Merck) or 0.5% (v/v) methanol.

2.2. Construction of plasmid pYPS1-delta

A plasmid pYPS1-delta for homologous recombination was constructed as below: (1) An ~0.5-kb DNA fragment containing N-terminal region of YPS1 gene was amplified using primers YPS1_NF and YPS1_NR (Table 1) in an automated thermal cycler (Biometra); (2) Another ~0.6-kb DNA fragment containing C-terminal region was obtained via the same method using primers YPS1_CF and YPS1_CR (Table 1); (3) The two fragments (YPS1_N and YPS1_C) were ligated reversely via fusion PCR (Wang et al., 2004); (4) The resulting ~1.1-kb fragment was subcloned into an ~2.2-kb BglII/SalI (Toyobo) plasmid fragment from pPICZαA (Invitrogen), containing pUC origin and *sh ble* gene (*Zeocin* resistance gene), by a T4 DNA ligase (Toyobo) to give the pYPS1-delta. Identification was carried out for the plasmid via restriction endonuclease digestion with BglII/SalI and ScaI, respectively, and subsequently via DNA sequencing performed by the Invitrogen Biotechnology Co., Ltd. (Shanghai).

2.3. YPS1 gene disruption

Transformation of *P. pastoris* GS115 strain with linearized pYPS1-delta fragment obtained by ScaI (Toyobo) digestion was carried out in a Gene Pulser (Bio-Rad) according to the modified electroporation method where the GS115 strain was pretreated with a buffer containing 10 mM Tris–HCl (pH 7.5), 100 mM LiAc, 10 mM DTT and 0.6 M sorbitol (Wu and Letchworth, 2004). YPS1 disruptant in *Zeocin*$^+$ transformants was selected by PCR analysis with primers p425 and p1371 (Table 1) annealing to the region covering the *sh ble* gene and two YPS1 gene fragments flanking the marker gene, and with primers p618 and p1178 (Table 1) annealing to the region displaced by the *sh ble* gene during homologous recombination.

2.4. Southern blot analysis

The YPS1 disruptant determined by PCR method was examined by Southern blot analysis for correct disruption of YPS1 gene. 20 μg genomic DNA was digested overnight with Asu II/SacI (Toyobo). The digest was then loaded on a 5 cm × 10 cm agarose gel for electrophoresis at 20 V for 3 h. The gel was denatured with 1.5 M NaCl, 0.5 M NaOH for 45 min, and neutralized with 1 M Tris–HCl (pH 8.0), 0.15 M NaOH (Amresco) for 30 min. Subsequently, the neutralized gel was assembled into the transfer set-up for transferring electrically the genomic DNA to a nitrocellulose (NC) membrane (Millipore). The NC membrane was baked at 80 °C in vacuum for 2 h. Prehybridization and hydridization with the NC membrane

Table 1
Oligonucleotide primers and probe used in this study.

Primers	Sequence (5′–3′)	Restriction site
YPS1_NF	GTATAGTAGTACTTTTCCGCTCAGCCAGATTTT	ScaI
YPS1_NR	TAACGTCGACAACTAGTGCTAGTTCCAACG	SalI
YPS1_CF	CGGAAGATCTCTCCTATGATTCGTCAAGAC	BglII
YPS1_CR	GCGGAAAAGTACTACTATACACACGCCGAGAAT	ScaI
p425	GTTCCCGCGTGAAGAGAGATATA	N/A[a]
p1371	TTCCACTGTAGCACCCCCAAAAT	N/A[a]
p618	TGCCACAGCCGTATCGGTAAGTT	N/A[a]
p1178	AGAGTTCCCTGGTCAGAACCCTT	N/A[a]
Probe	Biotin-CATCGTCCTCGACTTCCAGTGGAGACGAAGAAGGAGGGAGCTCCGCCAACAGGGTCCCCTTCAGCTACCT	N/A[a]

[a] N/A, not applicable.