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Linker length affects expression and bioactivity of the onconase fusion protein in *Pichia pastoris*

G.G. Yang[1,3], X.Y. Xu[1,3], Y. Ding[1,3], Q.Q. Cui[1,3], Z. Wang[2], Q.Y. Zhang[2], S.H. Shi[2], Z.Y. Lv[2], X.Y. Wang[2], J.H. Zhang[1,3], R.G. Zhang[1,3] and C.S. Xu[1,3]

[1]College of Life Science, Henan Normal University, Xinxiang, Henan, China
[2]Henan Xinxiang Hua Xing Pharmaceutical Factory, Xinxiang, Henan, China
[3]State Key Laboratory Cultivation Base for Cell Differentiation Regulation and Henan Bioengineering Key Laboratory, Henan Normal University, Xinxiang, Henan, China

Corresponding author: C.S. Xu
E-mail: cellkeylab@126.com

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ABSTRACT. The aim of this study was to analyze the effect of linker length on the expression and biological activity of recombinant protein onconase (ONC) in fusion with human serum albumin (HSA) in *Pichia pastoris*. Four flexible linkers with different lengths namely Linker L0, L1: $(GGGGS)_1$, L2: $(GGGGS)_2$, and L3:$(GGGGS)_3$ were inserted into the fusion gene and referred to as HSA-n-ONC, where N = 0, 5, 10, or 15. The sequence of the fusion gene HSA-ONC was designed based on the GC content and codon bias in *P. pastoris*; the signal peptide of albumin was used as the secretion signal. Gene sequences coding for the fusion protein with different linkers were inserted into pPICZα-A to form recombinant plasmids pPICZα-A/HSA-n-ONC, which were then transformed into *P. pastoris* X-33 for protein expression. Ideal conditions for expression of the fusion proteins were optimized at a small scale, using shake flasks before proceeding to mass production in 10-L fermenters. The recombinant fusion proteins

were purified by aqueous two-phase extraction coupled with DEAE anion exchange chromatography, and their cytotoxic effect on the tumor cell was evaluated by the sulforhodamine B assay. The results showed that the expressed amount of fusion proteins had no significant relationship with the length of different linkers and rHSA-0-ONC had no cytotoxic effect on the tumor cells. While rHSA-5-ONC and rHSA-10-ONC had a weak cytotoxic effect, rHSA-15-ONC could kill various tumor cells *in vitro*. In summary, the biological activity of the fusion protein gradually improved with increasing length of the linker.

Key words: Linker; Onconase; Human serum albumin; Fusion protein; Activity detection

INTRODUCTION

Onconase (ONC), also known as ranpirnase or P-30 protein, is an RNase that was originally extracted from the embryos of northern leopard frogs and has a strong cytotoxic effect on various tumor cells and solid tumors both *in vitro* and *in vivo*. ONC is the smallest member of the ribonuclease A (RNase A) family (Porta et al., 2008) and is considered unique since it has entered clinical trials (Darzynkiewicz et al., 1988; Ardelt et al., 1991). Once ONC is internalized and sorted to the cytosol, it could degrade the target tRNA to inhibit protein synthesis (Chang et al., 2010); however, the mechanism of its cytotoxicity is not completely understood. The company Timair (formerly known as Alfacell) has successively carried out I-III phase clinical trials of ONC on breast cancer, pancreatic cancer, non-small cell lung cancer, and malignant melanoma (Tian et al., 2010). ONC used in these clinical trials was purified from the embryos of *Rana pipiens* by a time-consuming and laborious process; however, the advent of recombinant DNA technology has provided efficient and cost-effective alternate sources of ONC (Wang et al., 2013). Earlier, recombinant ONC (rONC) was expressed as inclusion bodies in *Escherichia coli*, which is a widely used heterologous protein expression system. Because rONC cannot fold correctly and does not retain its activity, a complicated downstream process of refolding was necessary to obtain biologically active rONC (Wang et al., 2013). Highly active rONC could be expressed in *Pichia pastoris,* albeit at a low production rate (Zhao et al., 2009). Furthermore, human serum albumin (HSA), which has important biological functions and extensive clinical applications, is known to be expressed with high yield in the *P. pastoris* system and is therefore regarded as the ideal carrier (Kratz, 2008).

Linkers are usually inserted between fusion proteins to decrease their interaction especially at the protein folding stage (Arai et al., 2001; Ha et al., 2001; Hu et al., 2004). For human proteins, xenobiotic linkers are generally used, and the immunogenicity of the proteins has been shown to enhance if the linkers are particularly long, whereas short linkers lead to misfolding of the fusion proteins (Gustavsson et al., 2001; Le Gall et al., 2004). In this study, the nucleotide sequence coding for the five amino acids GGGGS was used as the linker resulting in the fusion gene *HSA-n-ONC*. Of the four linkers tested, three contained single or multiple copies of this sequence namely (GGGGS), $(GGGGS)_2$, and $(GGGGS)_3$ corresponding to N = 5, 10 and 15 amino acids, respectively. An end-to-end fusion without any linker sequence was also tested (N = 0). The fusion proteins rHSA-n-ONC were produced on a large-scale using 10-L fermenters, and the cytotoxic effect on the tumor cell was tested by sulforhodamine B (SRB assay).

MATERIAL AND METHODS

Strains, plasmids, and reagents

E. coli strain DH5α was used as the host for cloning the fusion gene *HSA-n-ONC*. *P. pastoris* strain X-33 and the protein expression vector pPICZα-A were purchased from Life Technologies (Carlsbad, CA, USA). Restriction enzymes, *Pme*I, *Bam*HI, and *Eco*RI, and protein ladder were purchased from Thermo Scientific (USA). The plasmid purification kit was purchased from Shanghai Sangon (Shanghai, China). The DNA sequences coding for the fusion proteins were synthesized by Sangon. Recombinant ONC was expressed and purified in our laboratory. Recombinant HSA was provided by Huaxing Pharmaceutical Factory (Henan, China).

Growth media

Yeast extract peptone dextrose (YPD) that contained 1% yeast extract (w/v), 2% peptone (w/v), and 2% glucose (w/v) was used as the culture medium.

Buffered minimal Glycerol-complex medium (BMGY) containing 1% yeast extract (w/v), 2% peptone (w/v), 1.34% yeast nitrogen base (YNB), 1% glycerol, and 0.4 µg/mL biotin, 10% potassium phosphate buffer, pH 6.0, was also used.

Low salt basic salt medium (LSBSM) with a trace element solution (PTM1) was used for high cell density cultivation in a 10-L bioreactor. LSBSM contained 6.5 g $(NaPO_3)_6$; 0.23 g $CaSO_4$; 4.55 g K_2SO_4; 3.73 g $MgSO_4.7H_2O$; 1.03 g KOH; and 40 g glycerol per liter. PTM1 contained 6.0 g $CuSO_4.5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4.H_2O$, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4.7H_2O$, 0.3 g biotin, and 5 mL concentrated H_2SO_4 per liter.

Construction of recombinant plasmids

The sequence coding the fusion protein HSA-n-ONC (N = 0, 5, 10, or 15) was designed based on the proportion of GC content and the codon bias of *P. pastoris*. The signal peptide of albumin was retained as the secretion signal of the fusion protein. DNA sequences coding for HSA and ONC mature proteins were synthesized with *Bam*HI and *Eco*RI restriction sites added to their 5'- and 3'-ends, respectively. The DNA sequences for the linker peptides containing 5, 10, or 15 amino acids corresponding to GGGGS, $(GGGGS)_2$, and $(GGGGS)_3$, respectively, were inserted into the fusion gene *HSA-n-ONC*. The recombinant expression plasmid pPICZα-A/*HSA-n-ONC*, which extracted in *E. coli* DH5α, was digested by *Bam*HI and *Eco*RI, and the restriction fragment was sequenced by Sangon (Shanghai, China).

Transformation into *P. pastoris* and selection of transformants

Competent *P. pastoris* X-33 cells were prepared according to the manufacturer instructions. After linearization of the recombinant plasmids pPICZα-A/*HSA-n-ONC* with *Pme*I, they were transformed into *P. pastoris* X-33 by the electroporation method. The transformed cells were grown on YPD with 100 µg/mL Zeocin. After incubation at 28°C for 2-3 days, positive clones were selected by a genomic PCR assay using common aldehyde oxidase 1 (*AOX1*) primers and verified by sequencing the PCR products.

Optimization of conditions for rHSA-n-ONC protein expression in *P. pastoris*

The positive clones of rHSA-n-ONC were cultured in 10 mL YPG medium overnight with shaking (250 rpm) at 28°C. These seed cultures were used to inoculate 100 mL BMGY medium (1% v/v) and cultured at 28°C and 280 rpm until the absorbance value (OD_{600}) reached approximately 20. At this point, few conditions were tested to determine the optimal induction time, culture pH, and final methanol concentration for protein expression. Cultures with pH values 5.5, 6.0, 6.5, 7.0, and 7.5 were induced with 1% methanol (v/v) for protein expression. Different incubation temperatures (20°, 23°, and 26°C) were assessed for optimal expression with shaking at 200 rpm. Methanol was added every 12 h to maintain the induction concentration and supernatants were collected at 240 h. The optimal fermentation conditions were selected based on the amount of recombinant protein produced. Protein concentration in the supernatants was determined by Bradford assay with bovine serum albumin as a standard, followed by SDS-PAGE (15%) analysis using 50-µL samples. Gels stained with Coomassie brilliant blue R-250 were scanned using a Typhoon FLA 7000 IP (GE health) and analyzed by the ImageQuant TL software.

Fed-batch fermentation for fusion protein expression in *P. pastoris*

To produce higher quantities of rHSA-n-ONC fusion proteins, the transformed clones were cultured in 4 L LSBSM medium in 10-L fermenters (Baoxing, Shanghai, China) at 28°C with shaking at 400 rpm and 0.2 NM^3/h ventilation for 12 h. Then, the rotational speed and ventilation were adjusted to 750 rpm and 1 NM^3/h, respectively. With the increase of dissolved oxygen (DO) in the zymotic fluid, glycerin which acted as carbon sources and provided nutrition for the *P. pastoris* was added until the OD_{600} reached 600. At the second stage, methanol supplemented with 2% PTM1 (v/v) was added when the glycerin in the zymotic fluid was depleted. For the next 10 days, the cultures were maintained at 20 DO, 0.25% methanol, 23°C, 750 rpm, and 1 NM^3/h ventilation. After 10 days, the fermentation process was terminated when the amount of rHSA-n-ONC reached their maximum in the culture supernatants. The fusion proteins in 10 µL supernatant was analyzed by SDS-PAGE.

Purification of rHSA-n-ONC

After large-scale fermentation, rHSA-n-ONC proteins in the supernatant were purified by aqueous two-phase extraction coupled with DEAE anion exchange chromatography. For 1 L supernatant, 385 g K_2HPO_4 was added and stirred thoroughly until completely dissolved. Subsequently, 372 mL ethanol was added, and the mixture was placed in a separatory funnel and placed overnight at -20°C, following which the solution divided into three phases. The upper phase was collected and concentrated by Vivaflow 200 modules (Sartorius Stedim Biotech) with a 30 kDa cut-off. The protein solution (50 mL) was applied on a X26 column (GE Healthcare) using the AKTA purifier system (GE Healthcare). The column was washed with 15 column volumes of disodium (hydrogen) phosphate buffer (25 mM, pH7.0), and the protein was eluted with disodium (hydrogen) phosphate buffer (pH7.0) supplemented with 0~1 M sodium chloride NaCl. The protein size and purity were assessed by SDS-PAGE.

Western blot

Each of the purified rHSA-n-ONC proteins (10 µg) was heat-denatured in Laemmli sample

loading buffer, separated by SDS-PAGE, and electrotransferred on a nitrocellulose membrane. Non-specific protein binding was prevented by treating the membrane with 5% non-fat dry milk for 2 h at room temperature. The membrane was then incubated overnight at 4°C with goat polyclonal anti-HSA (1:1000; Upstate, Charlottesville, VA, USA) and rabbit polyclonal anti-ONC (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary horseradish peroxidase-linked anti-rabbit (1:5000; Amersham Biosciences, Bucks, UK) or anti-goat (1:10000; Vector Laboratories, Burlingame, CA, USA) antibody was used for detection. Positive signals were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences) and captured on radiographic film (Hyperfilm ECL; Amersham Biosciences) through serial exposures.

Cytotoxicity assays

While the cell line 7402 (human liver cancer cells) was grown in RPMI-1640 medium with 10% fetal bovine serum (FBS), HeLa and RH-35 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37°C in a humidified atmosphere with 95 air and 5% CO_2. Between passages 30 and 50, the cells were seeded on 96-well plates at a concentration of 5 x 10^4 cells/well and cultured in DMEM with 10% FBS. The spent medium was replaced with medium supplemented with different concentrations of rHSA-n-ONC (0, 25, 50, and 100 μM) at 24 h, whereas PBS was used in the control group. After 24 h of treatment, the cells were fixed with 10% TCA followed by incubation at 4°C for thorough fixation. After 5X water washes in a stripwasher by alternate dispersion and aspiration, the plates were dyed with 100 μL 0.4% SRB (sulforhodamine B) for about 30 min, washed with 1% acetic acid to remove the excess, and air-dried overnight. To each well, 150 μL 10 mM Tris base solution was added, and the absorbance was measured at 490 nm wavelength. IC_{50} values were determined using three technical replicates each in triplicate by applying a non-linear 4P Hills-slope equation (Prism5 and SAS JMP software; variables top and bottom were set to 100 and 0, respectively).

RESULTS

Detection of recombinant expression plasmids

The recombinant plasmids pPICZα-A/*HSA-n-ONC* were constructed as described above and confirmed by enzyme digestion, PCR, and sequencing. A 5200-bp product was obtained when the recombinant plasmids were linearized by the restriction enzyme *Pme*I, whereas digestion with *Bam*HI and *Eco*RI resulted in two fragments of length 3000 and 2200 bp. PCR verification using universal *AOX* primers yielded the expected fragment of length 2500 bp (Figure 1).

Detection and expression of transformants

Recombinant plasmids pPICZα-A/*HSA-n-ONC* were linearized using *Pme*I and transformed into *P. pastoris* X-33 by electroporation. Transformants were selected using genomic PCR with *AOX1* primers (Figure 2A), and the PCR products verified by sequencing (Genewiz). Positive clones of rHSA-n-ONC were selected at random and verified for protein expression upon induction with methanol. The culture supernatants analyzed by SDS-PAGE at 240 h revealed a protein of approximately 77.3 kDa, which is the expected size of rHSA-n-ONC (Figure 2B).

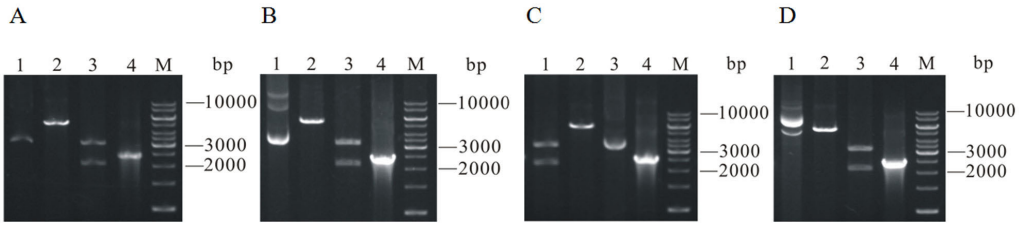

Figure 1. Verification of recombinant plasmids pPICZα-A/HSA-n-ONC. Electrophoresis of recombinant plasmids obtained from positive DH5α clones. **A.** pPICZα-A/HSA-0-ONC; **B.** pPICZα-A/HSA-5-ONC; **C.** pPICZα-A/HSA-10-ONC; **D.** pPICZα-A/HSA-15-ONC. *Lane 1*, recombinant plasmid; *lane 2*, recombinant plasmid digested with *Pme*I; *lane 3*, recombinant plasmid double-digested with *Bst*BI and *Eco*RI; and *lane 4*, PCR product obtained with AOX1 primers; M, DNA molecular weight standard (DL10000, Ding Guo, Beijing).

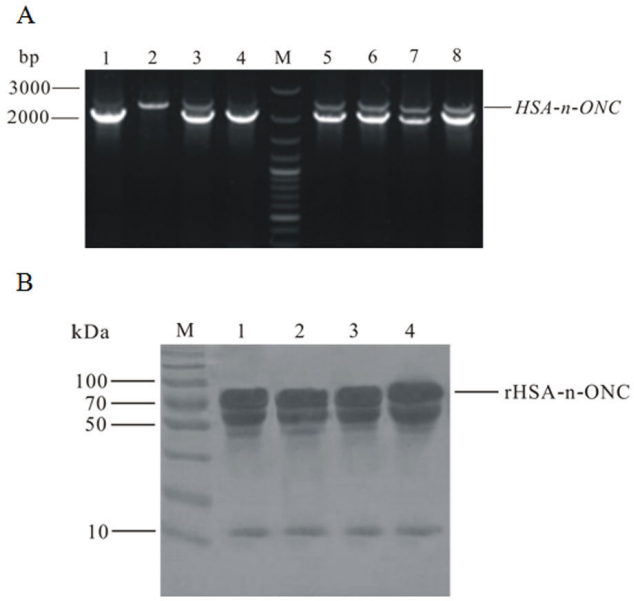

Figure 2. Identification of *Pichia pastoris* clones expressing recombinant HSA-n-ONC. **A.** Electrophoresis of genomic PCR with AOX1 primers. M, DNA ladder; *lanes 1-2*, rHSA-0-ONC; *lanes 3-4*, rHSA-5-ONC; *lanes 5-6*, rHSA-10-ONC; *lanes 7-8*, rHSA-15-ONC. **B.** SDS-PAGE analysis of recombinant HSA-n-ONC fusion proteins. M, protein marker; *lane 1*, rHSA-0-ONC; *lane 2*, rHSA-5-ONC; *lane 3*, rHSA-10-ONC; *lane 4*, rHSA-15-ONC.

Optimization of the conditions for protein expression at the shake flask scale

Production of secreted rHSA-n-ONC in *P. pastoris* was evaluated under different conditions, and the supernatant from each experiment was analyzed by SDS-PAGE. Induction temperature, culture pH, and methanol concentration required for effective induction of protein expression were investigated. Supernatant of cultures induced by 1% methanol were collected at 240 h. As shown in Table 1, the results showed that maximum level of rHSA-0-ONC was reached at 23°C and pH 6.5, whereas the levels of rHSA-n-ONC (N = 5, 10, 15) were maximum at 23°C and pH 7.0.

Table 1. Optimal conditions for the expression of recombinant HSA-n-ONC proteins.

Factor	rHSA-0-ONC	rHSA-5-ONC	rHSA-10-ONC	rHSA-15-ONC
T (°C)	23	23	23	23
pH	6.5	7.0	7.0	7.0
Concentration (mg/L)	212	228	209	235

Large-scale production of rHSA-n-ONC

The optimal culture conditions established for protein expression in *P. pastoris* at a small scale were applied for large-scale production. Maximum expression of rHSA-n-ONC in the 10-L fermenter using LS-BSM was achieved at 240 h with 0.25% methanol induction. The supernatants were analyzed by SDS-PAGE and the maximum concentration of rHSA-n-ONC was about 2 g/L (Figure 3). To minimize the technical errors from microarray analysis, each sample was tested at least three times, and the statistical analysis showed that there were no significant difference between the expression of rHSA-n-ONC.

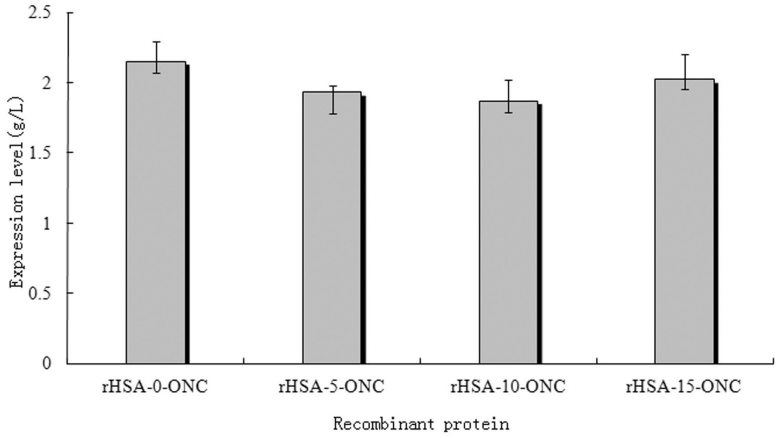

Figure 3. Effect of linker length on the expression of rHSA-n-ONC.

Purification of rHSA-n-ONC

Aqueous two-phase extraction coupled with DEAE anion exchange chromatography was employed to purify the rHSA-n-ONC fusion proteins in the culture supernatants, followed by SDS-PAGE (Figure 4A). The average purity was more than 95% and the average recovery was about 70% (Table 2). Results from immunoblotting assay using anti-HSA (Figure 4B) and anti-ONC (Figure 4C) antibodies revealed double antigenicity for the tested samples, thereby confirming the recombinant fusion proteins rHSA-n-ONC.

Cytotoxic activity of rHSA-n-ONC on tumor cells

The results from SRB assay to detect the effect of rHSA-n-ONC proteins on tumor cells showed that the intensity of the four different fusion proteins was different (Table 3). All data are

reported as means ± SD. The IC_{50} values of rHSA-n-ONC proteins were compared to that of rONC by using the Dunnett *post hoc* test; *P < 0.05 and **P < 0.01 were considered as statistically significant and very significant, respectively. While rHSA-0-ONC had no cytotoxic effect on the tumor cells, rHSA-5-ONC and rHSA-10-ONC had a weak effect, and rHSA-15-ONC was the most effective of all and could kill various tumor cells *in vitro*.

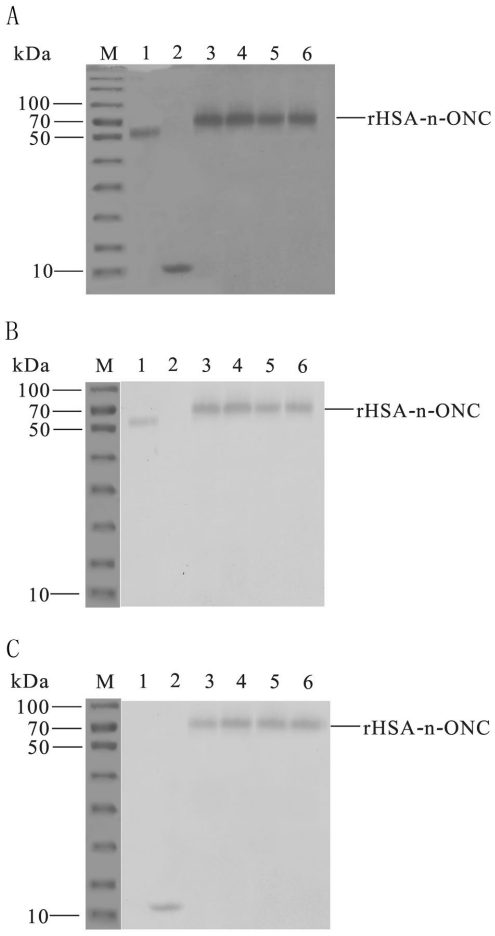

Figure 4. Verification of purified rHSA-n-ONC proteins. **A.** SDS-PAGE analysis of purified rHSA-n-ONC; **B.** western blotting with anti-HSA antibody; **C.** western blotting with anti-ONC antibody; M, protein marker; *lane 1*, rHSA; *lane 2*, rONC; *lane 3*, purified rHSA-0-ONC; *lane 4*, purified rHSA-5-ONC; *lane 5*, purified rHSA-10-ONC; *lane 6*, purified rHSA-15-ONC.

Table 2. Characteristics of purified recombinant HSA-n-ONC proteins.

Condition	rHSA-0-ONC	rHSA-5-ONC	rHSA-10-ONC	rHSA-15-ONC
NaCl (mM)	65	75	75	75
Purity (%)	95	95	95	95
Yield (%)	73	75	71	76

Table 3. Cytotoxic effect of ONC on 7402, HeLa, and RH-35 cells.

Cell lines	rHSA-0-ONC	rHSA-5-ONC	rHSA-10-ONC	rHSA-15-ONC	rONC
7402	inactive	>100	>100	18.56 ± 0.49**	6.55 ± 0.27
HeLa	inactive	44 ± 2.12**	38 ± 1.68**	16.17 ± 0.58**	2.33 ± 0.13
RH-35	inactive	>100	57 ± 3.44**	19.44 ± 0.75**	7.78 ± 0.38

IC_{50} values are reported as mean ± standard deviation (μM). **P < 0.01.

DISCUSSION

Onconase (ONC) has shown promising anti-tumor effect on many solid tumors and hence, is being marketed in Europe and America. However, the commercially available ONC is being extracted from leopard frog *Rana pipiens*, which is a great limitation to its mass production. In this study, the effect of flexible linkers on the expression and activity of recombinant ONC in fusion with human serum albumin (HSA-n-ONC) was analyzed, since HSA-fused proteins are expected to have higher expression and stronger activity in *P. pastoris* (Kratz, 2008).

Theoretically, a fusion protein would have the functions of both proteins; however, their function and activity have been reported to be changed (Geng et al., 2006; Tranchant et al., 2006), or lost, and in some cases, the proteins were not even expressed (Doi and Yanagawa, 1999; Karp and Oker-Blom, 1999; Hong et al., 2006). Only a few proteins synthesized by fusion have exhibited the expected function (Lu et al., 2006; Orita et al., 2007; Seo et al., 2008). It is generally recognized that closer spatial distance between the fused proteins affects their folding, function, and expression. Flexible linkers are typically incorporated in cases where the fused domains require a certain degree of movement or interaction (Kim et al., 2000; Blazyk and Lippard, 2004; Jiang et al., 2005; Wang et al., 2007; Lu and Feng, 2008). Most commonly used flexible linker sequences primarily consist of Gly and Ser residues ("GS" linker) (Huston et al., 1988; Kavoosi et al., 2007; Hölsch and Weuster-Botz, 2010). $(G_4S_1)_n$ is one such widely used sequence. While long flexible linkers might lead to immune responses, those with no more than 15 amino acids would have little effect on the folding and function of the proteins.

In protein expression systems, differences between the host and exogenous genes often lead to gene silencing or low expression levels, which can be overcome by optimization of the exogenous gene codon (Abad et al., 2011; Mellitzer et al., 2012; Sygmund et al., 2012). Both codon preferences and optimization of the fermentation process are common ways to improve the yield and activity of heterologous proteins (Park et al., 2012; Spatz et al., 2013). In this study, flexible linkers with different lengths were inserted between *HSA* and *ONC* genes to increase the expression levels of rHSA-n-ONC (N = 0, 5, 10, and 15). Our results indicated that the best culture temperature in the shake flask was 23°C for all the four proteins, and the best pH values were 6.5 for end-to-end fusion protein, and 7.0 for the ones with linkers. The highest concentration of HSA-n-ONC (N = 0, 5, 10, 15) when expressed in 10-L bioreactors were 2.15, 1.93, 1.87, and 2.02 g/L, respectively. Recombinant HSA-n-ONC proteins with ≥95% purity could be recovered efficiently (≥70%) from supernatants of large scale cultures that were purified by a combination of aqueous two-phase extraction and DEAE anion exchange chromatography.

Evaluation of the lethal effect of rHSA-n-ONC on cancer cells by the SRB assay revealed that the length of flexible linkers had an effect only on the activity of rHSA-n-ONC but not on its expression. While the bioactivity of rONC was the strongest, the cytotoxicity of rHSA-n-ONC increased with linker length. Improper assembly due to spatial interference of component domains

might have led to the loss of antitumor activity in the end-to-end fusion protein rHSA-0-ONC; similar cases have also been reported previously (Karp and Oker-Blom, 1999; Hong et al., 2006). The bioactivity of rHSA-5-ONC was similar to that of rHSA-10-ONC but stronger than that of rHSA-0-ONC. Although rHSA-10-ONC was expected to be more cytotoxic than rHSA-5-ONC, no significant difference was observed between them. The reason might be that the spatial distance between $(GGGGS)_2$ was not exactly double that of GGGGS, and the effect of a single copy was not steady. Nevertheless, rHSA-15-ONC had a substantial lethal effect on three kinds of cancer cells similar to that observed with rONC; however, its activity was much lesser than that of rONC. It might be assumed that the spatial distance occupied by $(GGGGS)_3$ would be considerably longer compared to that occupied by GGGGS and $(GGGGS)_2$. Taken together, these results demonstrate that flexible linkers have a significant effect on the bioactivity of fusion proteins.

Short linkers $(GGGGS)_n$ ($N \leq 6$) have been used successfully in many fusion proteins, but they also have deficiencies such as vulnerability to proteases, which in turn leads to the degradation of proteins and the inability of isolated short linkers to avoid spatial interference of domains (Maeda et al., 1997; Arai et al., 2001, 2004). Although many researchers have focused on the design of flexible linkers, an effective method is yet to be found. Therefore, it is necessary to consider the structure and composition of flexible linkers in designing fusion proteins for them to be bioactive and effectively expressed.

Conflicts of interest

The authors declare no conflict of interest.

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