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Metabolic engineering of *Pichia pastoris* for production of hyaluronic acid with high molecular weight

Q1 Euijoon Jeong, Woo Yong Shim, Jung Hoe Kim*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 291 Daehak-ro (373-1 Guseong-dong), Yuseong-gu, Daejeon 305-701, Republic of Korea

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ABSTRACT

The high molecular weight (>1 MDa) of hyaluronic acid (HA) is important for its biological functions. The reported limiting factors for the production of HA with high molecular weight (MW) by microbial fermentation are the insufficient HA precursor pool and cell growth inhibition. To overcome these issues, the *Xenopus laevis xhasA2* and *xhasB* genes encoding hyaluronan synthase 2 (*xhasA2*) and UDP-glucose dehydrogenase (*xhasB*), were expressed in *Pichia pastoris* widely used for production of heterologous proteins. In this study, expression vectors containing various combination cassettes of HA pathway genes including *xhasA2* and *xhasB* from *X. laevis* as well as UDP-glucose pyrophosphorylase (*hasC*), UDP-N-acetylglucosamine pyrophosphorylase (*hasD*) and phosphoglucose isomerase (*hasE*) from *P. pastoris* were constructed and tested. First, HA pathway genes were overexpressed using pAO815 and pGAPZB vectors, resulting in the production of 1.2 MDa HA polymers. Second, in order to decrease hyaluronan synthase expression a strong AOX1 promoter in the *xhasA2* gene was replaced by a weak AOX2 promoter which increased the mean MW of HA to 2.1 MDa. Finally, the MW of HA polymer was further increased to 2.5 MDa by low-temperature cultivation (26 °C) which reduced cell growth inhibition. The yield of HA production by the *P. pastoris* recombinant strains in 1 L of fermentation culture was 0.8–1.7 g/L.

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1. Introduction

Hyaluronic acid (HA) is a natural linear polysaccharide composed of repeating units of (1,4)-β-linked glucuronic acid and (1,3)-β-linked N-acetylglucosamine. This high molecular weight (5 KDa–10 MDa) biopolymer is abundant in vertebrate tissues including the skin, eyes, umbilical cord, and joints (Kogan et al., 2007; Olczyk et al., 2008). HA is an essential functional component in the body which has a number of applications in medicine, cosmetics, and specialty foods (Moskowitz, 2000; Chong et al., 2005; Allison and Grande-Allen, 2006). The global market for HA was estimated at over 1.4 billion USD in 2010 (Liu et al., 2011). HA applications depend on its molecular weight (MW), and HA with the MW over 1 MDa is used for clinical and cosmetic applications (Guillaumie et al., 2010).

Traditionally, high-MW HA is chemically extracted from animal waste such as rooster combs or umbilical cords; however, these resources are limited. For HA mass production, microbial fermentation using group A or group C streptococci which naturally produce HA for protection from host immune responses, is employed, although this method has limitations due to safety concerns and HA quality (Chong et al., 2005; Kogan et al., 2007). The MW of HA produced by streptococcal strains is in the range of 0.8–1.5 MDa which is relatively small compared to that of the HA extracted from animal tissues (over 3 MDa). Therefore, several groups have attempted to establish HA production based on recombinant bacteria including *Escherichia coli* (Yu and Stephanopoulos, 2008), *Bacillus subtilis* (Widner et al., 2005), *Lactococcus lactis* (Chien and Lee, 2007), and *Agrobacterium* sp. (Mao and Chen, 2007) engineered to overexpress streptococcal hyaluronan synthase (*hasA*). HA production using these recombinant strains helps avoid the problem of pathogenicity and hyaluronidase expression. However, HA with high MW around 1 MDa comparable to that of the streptococci-derived HA was synthesized only by the recombinant *B. subtilis* (Widner et al., 2005), while the production of HA with MW over 2 MDa has not been established yet.

In this study, we attempted to establish the production of recombinant HA with MW >2 MDa, similar to that of streptococcal HA. The increase of the HA chain length is essential for clinical and other applications, but it is difficult to obtain recombinant HA with the MW over 1.0 MDa. Two limiting factors for the production of high MW HA are the shortage of precursors and inhibition

* Corresponding author. Tel.: +82 42 350 2654; fax: +82 42 350 2690.
E-mail address: kimjh@kaist.ac.kr (J.H. Kim).

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Table 1
Primers used in this study.

Primer name	Primer sequence (5′→3′)	Digestion site
C(*ugp*)-F	CTCGAGAAAAATGTCTGCTTACCAGTCC	*Xho* I
C(*ugp*)-R	TCTAGATTAGTGCTCCAAGATAGTCAAGTTTC	*Xba* I
D(*uap*)-F	CTCGAGAAAAATGTCTTTGGAGCAGTAC	*Xho* I
D(*uap*)-R	TCTAGATTAGATAACGTCACCGTTCTTGAA	*Xba* I
E(*pgi*)-F	CTCGAGAAAAATGCCATCTTTGTTG	*Xho* I
E(*pgi*)-R	TCTAGATTAGGCCCAAGCCTTGAAC	*Xba* I
B(*xugd*)-F	CTCGAGAAAAATGTTCCAGATCAAGAA	*Xho* I
B(*xugd*)-R	TCTAGATTAGACTCTCTGCTTCTTGTGTGG	*Xba* I
A(*xhas2*)-F	CTCGAGAAAAATGCACTGTGAGAGATT	*Xho* I
A(*xhas2*)-R	TCTAGATTAGGCCAAAACCAAGTCG	*Xba* I
rtC(*ugp*)-F	GATCGACCCAGCTAGATTTGGTGC	–
rtC(*ugp*)-R	GAACACCCTTACCGAACTGAACGTTAC	–
rtD(*uap*)-F	GAAGTTCGGTTCCTTGGAGGTTAAGAG	–
rtD(*uap*)-R	GGAAGCACCGTTTTCTTTGATCCAC	–
rtE(*pgi*)-F	CATCTTGACTCAGAAGATCACTCCAGCTAC	–
rtE(*pgi*)-R	CTTTTGAACAGCCTTAGCCAAAACCTTAC	–
rtB(*xugd*)-F	GACATGTTCAAAGAGTTGGACTTCAACAG	–
rtB(*xugd*)-R	CAACCTTCTTACCGATAGTCTCAACCTG	–
rtA(*xhas2*)-F	GAACATCTTGTTGTTCTTGTTGACTGTTC	–
rtA(*xhas2*)-R	CTTAGCTGGCAACAAGGATGACATGTAC	–
rtActin-F	CCTGAGGCTTTGTTCCACCCATCT	
rtActin-R	GGAACATAGTAGTACCACCGGACATAACGA	
pAO815-seq-F	GTATTGTGAAATAGACGCAGATCG	–
pAO815-seq-R	CGGTGCCTGACTGCGTTAG	–
pGAPZB-seq-F	CATGAGATTATTGGAAACCACCAGAAT	–
pGAPZB-seq-R	CAACTTGAACTGAGGAACAGTCATGTCTA	–

of recombinant cell growth (Chong et al., 2005; Kogan et al., 2007). To overcome these issues, we investigated the use of recombinant methylotrophic *Pichia pastoris*, which is widely used as a production platform for heterologous proteins and has a big pool of sugar precursors compared to bacterial hosts. First, we constructed *P. pastoris* strains expressing *Xenopus laevis* hyaluronan synthase, the enzyme which was reported to synthesize HA polymers with the MW of 20 MDa *in vitro* (Pummill et al., 1998). Second, we enriched both the pool of sugar precursors and the ratio of the HA precursor pool to the total activity of hyaluronan synthase. Finally, low-temperature cultivation was employed in order to overcome cell growth inhibition.

2. Materials and methods

2.1. Strains and media

E. coli DH5a (RBC) was used for gene cloning. *E. coli* cells were grown on LB agar or broth with ampicillin at 50 μg/mL or zeocin at 25 μg/mL. *P. pastoris* GS115 (Invitrogen) was used for as an expression host. For genetic manipulation of *P. pastoris*, YPD medium with zeocin at 100 μg/mL and YNB medium were used.

2.2. PCR gene amplification, plasmid vectors, and construction of *P. pastoris* strains

The following HA pathway genes were amplified by PCR and cloned into the pGEM-T plasmid: the *ugp*, *uap*, and *pgi* genes of *P. pastoris*. The *xhasA2* and *xudp* genes of *X. laevis* were synthesized using the most preferred codons in the codon usage database (http://www.kazusa.or.jp/codon). Primers used for cloning and sequencing of the *xhasA2*, *xhasB*, *hasC*, *hasD* and *hasE* genes and for construction of the pAO815 and pGAPZB plasmids are listed in Table 1. The plasmids pAO815 and pGAPZB (Invitrogen) were used to construct six expression cassettes containing the HA pathway genes which were introduced into *P. pastoris* for HA synthesis. The multiple cassettes were constructed using *Bgl*II and *Bam*HI sites. Each vector containing the HA pathway gene was digested by *Bgl*II and *Bam*HI and ligated with a linear vector containing the *xhas2* gene digested by *Bam*HI. The constructed recombinant *P. pastoris* GS115 strains are listed in Table 2.

Table 2
Combinatorial strains constructed in this study.

Strains	pAO815 (MeOH induced)	pGAPZB (constitutive)
EJ	AB	–
EJP-D	AB D	BCDE
EJP-CD	AB CD	BCDE
EJP-CDE	AB CDE	BCDE
EJW	A′B CDE	–
EJWP-CD	A′B CD	BCDE
EJWP-CDE	A′B CDE	BCDE

A, *xhasA2*; B, *xhasB*; C, *hasC*; D, *hasD*; E, *hasE*.
A′ includes a weak promoter.
EJ strain expresses *xhasA2* and *xhasB*; EJP series strains overexpress HA pathway genes; EJW strain has a reduced *xhasA2* expression; EJWP series strains overexpress HA pathway genes and have a reduced *xhasA2* expression.

2.3. Analysis of mRNA expression by real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of 1.0 μg of RNA to cDNA was performed using AccuPower RT-PCR PreMix (Bioneer). Each strain cDNA was used as template for real-time PCR performed using the MyiQ™ real-time detection system (Bio-Rad, Hercules, CA, USA). PCR conditions included initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. The 20-μL reaction mixture contained 2× Fast Start SYBR Green Mater (Roche, Mannheim, Germany), 10 pmol forward and reverse primers, and 5 μL of 1:10 diluted cDNA template. Specific primer pairs (Table 1) were used to analyze transcript levels of each enzyme which were normalized to the expression level of *P. pastoris* actin amplified using primers. Raw data obtained by real-time PCR were analyzed using the iQ™5 Optical System software (Bio-Rad).

2.4. Enzyme assays

Cells were harvested from fermentation broth and pelleted at 3000 rpm, 4 °C for 5 min. The supernatant was discarded, and the pellet was washed twice in PBS. Finally, the pellet was resuspended in wash buffer containing protease inhibitor and mixed with glass beads. Tubes were chilled on ice for 3 min prior to disruption in a Mini Bead Beater (Bio-Rad) through 6 cycles of 30 s beating followed by 1.5 min on ice between cycles. Then, the sample was centrifuged (12,000 rpm, 4 °C for 10 min), and the supernatant was obtained. Protein content of the extracts was determined using protein assay kit (Bio-Rad).

The activity of HasA was measured by incubating cell extract with UDP-GlcNAc and UDP-GlcA for 1 h at 37 °C. The reaction was stopped by immersion in boiling water, before addition of 0.1% SDS to free HA molecules from membranes and measuring HA produced with the modified carbazole assay. The specific activity of 1 unit/mg of dry cell HA synthase is equivalent to 1 mg of HA generated/min/mg of dry cell.

HasB, HasC, and HasE activities were measured in NADH/NADPH-linked enzyme assays at room temperature. HasB activity was measured based on the conversion of UDP-Glc to UDP-GlcA. HasC activity was measured based on conversion of glucose 1-phosphate to UDP-Glc coupled to conversion of UDP-Glc to UDP-GlcA using excess UDP-Glucose dehydrogenase. HasE activity was measured from the conversion of fructose 6-phosphate to glucose 6-phosphate coupled to conversion of glucose 6-phosphate to 6-phosphogluconate using excess glucose-6-phosphate dehydrogenase. Specific activities were expressed as nanomoles of substrate converted into product in 1 min for 1 mg of

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