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ORIGINAL RESEARCH PAPER

Temperature-controlled molecular weight of hyaluronic acid produced by engineered *Bacillus subtilis*

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Abstract To produce high-, medium- and low-molecular-weight hyaluronic acid (HA) at different temperatures using engineered *Bacillus subtilis* expressing hyaluronidase (HAase) from leech. By overexpressing the HAase gene *hya* in the HA-producing strain WmB using temperature-sensitive plasmid pKSV7, the engineered strain WmB-PYh produced HA with different molecular weights (8.61 kDa at 32 °C, 0.615 MDa at 42 °C, and 6.19 MDa at 47 °C). In this study, the molecular weight of HA was regulated by using leech HAase expressed from a temperature-sensitive plasmid. We thus obtained different molecular weight HAs by using a single bacterial strain at different culture temperatures.

Keywords *Bacillus subtilis* · Hyaluronic acid · Hyaluronidase · Molecular weight · Microbial engineering · Temperature-sensitive expression

Introduction

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan that has been applied in cosmetic, food and pharmaceutical industries (Kogan et al. 2007). N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcUA) monomers polymerize via β-1,3- and β-1,4-glycosidic bonds to form HA chains, the length of which determines the molecular weight (MW), and different HAs have different functions (Stern et al. 2006). High-molecular-weight (HMW) HAs (MW > 2 MDa) have good viscoelasticity, strong rigidity, and maintain their properties for a long time. HMW HAs can be used in ophthalmic surgery and are useful for wound healing. Medium-molecular-weight (MMW) HAs (MW 0.1−1 MDa) display good moisture retention and lubricity, and they are suitable for soft tissue filling, deep wrinkle removal, and drug release. Low-molecular-weight

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(LMW) HAs (MW < 10 kDa) have superior permeability and cell penetration properties, can inhibit tumor proliferation, promote bone formation and angiogenesis, and be used in immunomodulation (Stern et al. 2006; Toole et al. 2008).

In recent years, heterologous HA-producing strains with clear backgrounds have become established as an attractive alternative for HA production due to the high pathogenic risk and paucity of DNA manipulation techniques for *Streptococcus* species (Blank et al. 2005). Many hosts, including *Bacillus subtilis* (Widner et al. 2005), *Lactococcus lactis* (Chien and Lee 2007), *Agrobacterium* sp. (Mao and Chen 2007), *Escherichia coli* (Yu and Stephanopoulos 2008), *Pichia pastoris* (Jeong et al. 2014) and *Corynebacterium glutamicum* (Cheng et al. 2019), have been engineered extensively for HA production. *B. subtilis*, an established "Generally Recognized as Safe" strain, is considered ideal for metabolic engineering (Commichau et al. 2015), and has been studied for HA production.

Leech hyaluronidase (LHAase) can specifically hydrolyze HA by acting on the non-reducing end of HA and cleaving the β-1,3-glycosidic bond, thereby decreasing the molecular weight of HA (Linker et al. 1960). In addition, exogenous expression of recombinant LHAase in *B. subtilis* has no risk of animal cross-infection (Jin et al. 2014). Therefore, LMW HAs for clinical medical treatment can be obtained using recombinant leech HAase. However, industrial processes for producing HAs with different MWs are complex.

Different MW HAs have been collected by modifying the ribosome binding site (RBS) of *hya* (encoding LHAase) in different engineered strains (Jin et al. 2016). In the present study, the temperature-sensitive vector pKSV7 was used to overexpress LHAase in *B. subtilis*. We regulated the expression of LHAase by controlling the temperature. This provides a novel strategy for producing high-, medium- and LMW HAs using a single bacterial strain at different culture temperatures (Fig. 1).

Materials and methods

Media and culture conditions

Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used for seed cultivation, with ampicillin (100 mg/L), ergomycin (50 mg/L) and tetracycline (50 mg/L) added as necessary. *E. coli* and *B. subtilis* strains were cultured overnight at 37 °C. Fermentation medium (20 g/L sucrose, 3 g/L $(NH_4)_2SO_4$, 6.5 g/L KH_2PO_4, 4.5 g/L Na_2HPO_4, 2 g/L sodium citrate, 3 g/L $MgSO_4 \bullet 7H_2O$, and 0.5 g/L $CaCl_2 \bullet 2H_2O$) and 6 mL/L of a trace metallic elements solution (100 g/L citric acid, 20 g/L $FeSO_4 \bullet 7H_2O$, 5 g/L $MnSO_4 \bullet H_2O$, 2 g/L $CuSO_4 \bullet 5H_2O$ and 2 g/L $ZnCl_2$) was used for HA production. *B. subtilis* strains were incubated at 32 °C, 42 °C and 47 °C.

Strains and plasmid construction

E. coli DH5α chemically competent cells were prepared as described previously (Russell and Sambrook 2001) and used as hosts for plasmid construction. The *hya* sequence was downloaded from NCBI and synthesized by Genewiz (Tianjin, China). The signal peptide of the *yweA* gene was amplified from *B. subtilis* WB600 and connected to the 5′- end of *hya* to form a fused *yweA–hya* fragment. The *yweA–hya* fragment was subsequently inserted into the temperature-sensitive plasmid pKSV7, resulting in plasmid pKSV7-Yh. Then, the strong promoter P_{43} was placed in front of *yweA–hya* and the new fragment P_{43}–*yweA–hya* was linked to pKSV7 to generate pKSV7-PYh. The constructed plasmids were electrotransformed into *B. subtilis* WB600 and WmB (Li et al. 2019). The *B. subtilis* strains and plasmids used in this study are listed in Table 1, and the primers used are listed in Supplementary Table 1.

Hyaluronidase activity analysis

LHAase activity was quantified using the 3,5-dinitrosalicylic acid (DNS) colorimetric spectrophotometric method (Jin et al. 2014). Controls comprising the fermentation supernatant of *B. subtilis* Wm were prepared and analyzed in an identical manner.

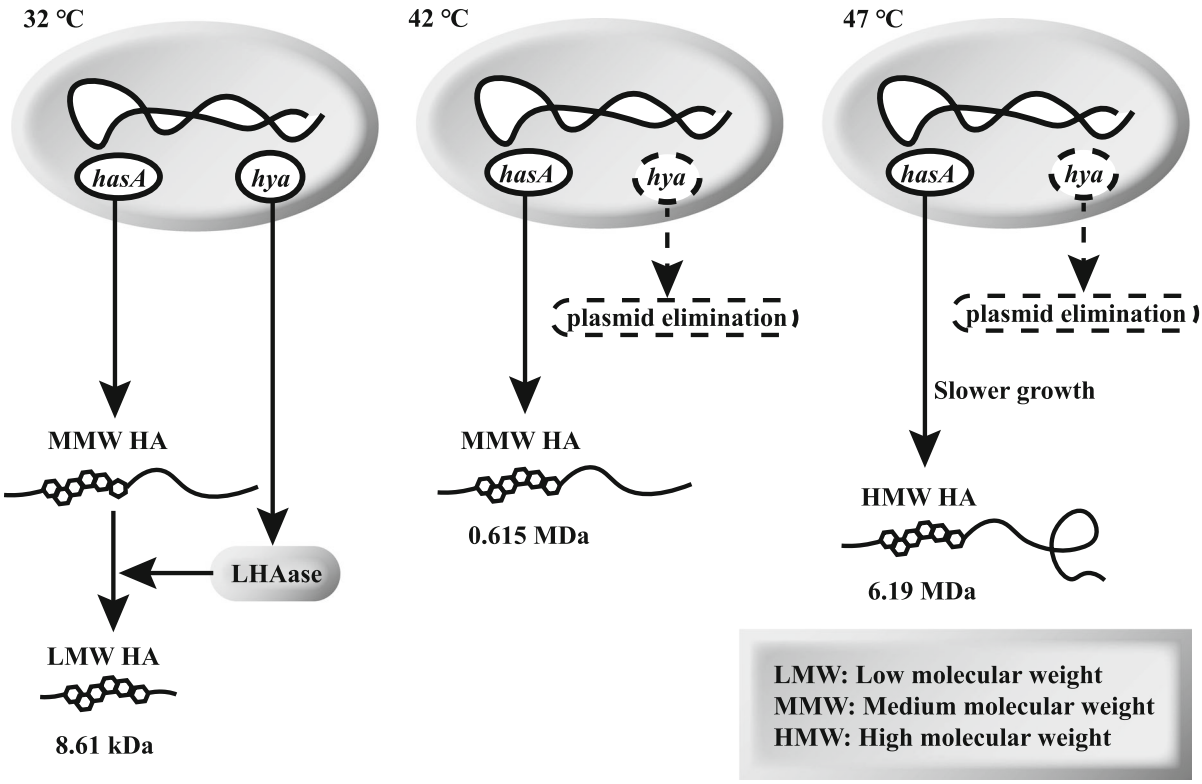

Fig. 1 Schematic diagram illustrating HAs with different molecular weights produced by *Bacillus subtilis* strain WmB-PYh at 32 °C, 42 °C and 47 °C

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source
E. coli		
DH5α	*sup E44 Δlac*U169(*ϕ 80lac ZΔM15*) *hsd R17 recAI endAI gyrA96 thi-1 relA*	Laboratory stock
B. subtilis		
B. subtilis WB600	*B. subtilis* gene-deficient strain (*trp*C2, Δ*nprE*, Δ*aprE*, Δ*nprB*, Δ*bpr*, Δ*mpr*, Δ*epr*)	(Wu, Lee et al. 1991)
Wm	WB600Δ*upp*Δ*sigF*	(Li, Li et al. 2019)
WmB	WB600Δ*upp*Δ*sigF*, *hasA, hasB* and *hasC*	(Li, Li et al. 2019)
Wm-pKSV7	Wm, pHT01 empty vector	This work
Wm-Yh	Wm, Pgrac-ftsZ	This work
Wm-PYh	Wm, Pgrac-mreB	This work
WmB-PYh	WmB, Pgrac-mreB	This work
plasmid		
pKSV7	Temperature-sensitive replication pET194ts, Amp^R, Erm^R, *E. coli-B. subtilis* shuttle vector	(Kang, Ruiyang et al. 2013)
pKSV7-Yh	YweA-hya	This work
pKSV7-PYh	P43-YweA-hya	This work

[a]*Amp*^{R} ampicillin resistance cassette, *Erm*^{R} erythromycin resistance cassette

HA quantification

HA fermentation products were collected as described previously (Li et al. 2019), and the HA titer was routinely determined using a modified uronic acid carbazole reaction (Bitter and Muir 1962). As a control, *B. subtilis* strain WB600 was treated in the same way and the value (background signal) was subtracted.

HA molecular weight measurement

The MW of HA was measured with a multi-angle laser light scattering and size exclusion chromatography system as described previously (Li et al. 2019).

Results and discussion

Expression of hyaluronidase in *B. subtilis* Wm

Due to the specificity of LHAase for hydrolyzing HA, it can be used to lower the MW of HA (Jin et al. 2016). To accomplish high-level extracellular production of LHAase in *B. subtilis*, we fused the LHAase gene *hya* with the signal peptide of *yweA* in the temperature-sensitive plasmid pKSV7. In theory, *hya* can be expressed at 32 °C, but there should be no expression of *hya* at 42 °C due to elimination of plasmid at this temperature (Smith and Youngman 1992). We constructed the engineered strain Wm-Yh expressing LHAase from the spore deletion strain Wm (Li et al. 2019), as confirmed by gel electrophoresis (Supplementary Fig 1). To verify LHAase activity, the resulting Wm-Yh strain was cultivated in flasks and the fermentation supernatant was analyzed by the DNS method (Fig. 2a). The reaction solution comprising Wm-Yh supernatant became reddish brown, while it was still yellow in solution containing Wm supernatant. The results indicated that Wm-Yh could produce active LHAase. The mean hyaluronidase activity of Wm-Yh was 2.04×10^4 U/mL (Fig. 2b). The activity was higher than that for enzyme expressed in *Pichia pastoris* (1.20×10^4 U/mL) (Jin et al. 2014).

Enhancement of hyaluronidase activity in *B. subtilis* Wm

To further promote the expression of LHAase, the strong promoter P_{43} was placed in front of the fused *yweA-hya* fragment in Wm, resulting in strain Wm-PYh. Gel electrophoresis results are shown in Supplementary Fig. 2. The wild-type RBS of *hya* has been verified to produce the lowest MW HAs (Jin et al. 2016). Compared with Wm-Yh, the product of the DNS reaction was darker for Wm-PYh (Fig. 2a). The mean HAase activity of Wm-PYh was 4.77×10^4 U/mL, significantly higher than that of Wm-Yh (Fig. 2b). These results indicated that the *hya* of pKSV7-PYh could be expressed at high levels in *B. subtilis*. Accordingly, plasmid pKSV7-PYh was chosen and engineered to explore its effect on HA MWs.

Production of LMW and MMW HA by *B. subtilis* WmB-PYh

Recombinant *B. subtilis* typically produce MMW HAs (Widner et al. 2005; Westbrook et al. 2018; Li et al. 2019). However, HAs with different MWs have different functions, and recombinant LHAase can be expressed to expand the MW range of HAs (Jin et al. 2016). Herein, the temperature-sensitive plasmid pKSV7-PYh was electrotransformed into the HA-producing WmB strain to construct WmB–PYh, and this strain produced LMW (8.61 kDa) and MMW (0.615 MDa) HAs at 32 °C and 42 °C, respectively (Fig. 3a). Additionally, WmB not expressing LHAase produced MMW HAs at both 32 °C (0.392 MDa) and 42 °C (0.678 MDa; Fig. 3a). Compared with WmB, there were no differences in HA MW for WmB-PYh at 42 °C, at which temperature pKSV7-PYh was eliminated. These results suggest that it may be more convenient and practical to produce LMW and MMW HAs by rationally controlling the expression of LHAase than by employing previously reported strategies (Jin et al. 2016). Additionally, the HA titers of WmB (4.01 g/L and 2.66 g/L) and WmB-PYh (4.25 g/L and 2.77 g/L) were not significantly different at 32 °C and 42 °C (Fig. 3b). This indicates that this strategy only affects the MW of HA.

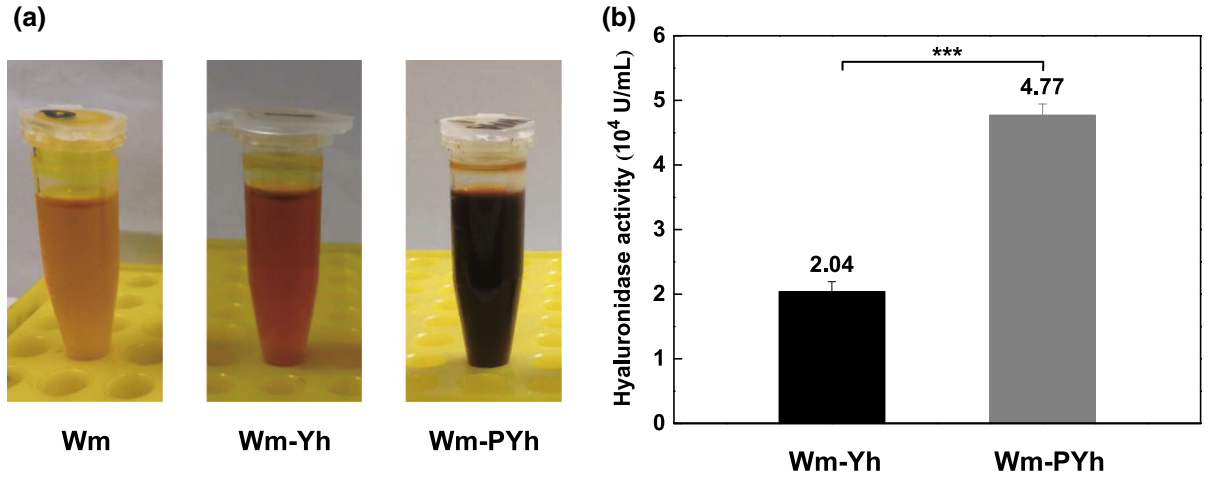

Fig. 2 Determination of leech hyaluronidase activity by the DNS method. **a** Photos of DNS and hyaluronidase color reactions following fermentation by Wm, Wm-Yh and Wm-PYh strains. **b** Hyaluronidase activity following fermentation by Wm-Yh and Wm-PYh. Data are averages of three independent experiments, and error bars represent standard deviation (SD; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, no significance)

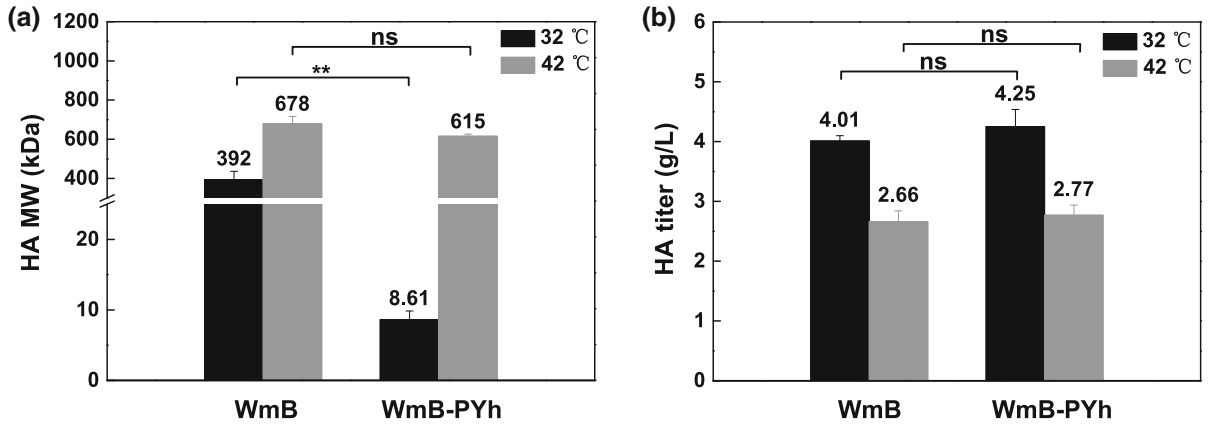

Fig. 3 HA MWs (**a**) and titers (**b**) for WmB and WmB-PYh at 32 °C and 42 °C. Data are averages of three independent experiments and error bars represent ± SD ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, no significance)

Further increasing the MW of HAs produced by *B. subtilis* WmB-PYh

Since HA is a growth-associated product (Huang et al. 2006), the MW can be increased by decreasing the HA-producing bacterial cell density using the oxygen vector n-heptane (Westbrook et al. 2018). Moreover, our previous research showed that WmB can produce HMW HAs (6.94 MDa) when the culture temperature is increased to 47 °C (Li et al. 2019). Similarly, WmB-PYh produced HMW HAs (6.19 MDa) at 47 °C in the present work (Fig. 4a). Compared with WmB (1.72 g/L), the HA titer of WmB-PYh (1.88 g/L) was not significantly different at 47 °C (Fig. 4b). These results demonstrate that lower cell density is conducive for extension of HA chains.

Conclusion

In this study, we developed a novel strategy to produce high-, medium- and LMW HAs using WmB-PYh. By adjusting the temperature to control the presence of the pKSV7-PYh plasmid, WmB-PYh produced HAs with different MW (8.61 kDa at 32 °C, 0.615 MDa at 42 °C and 6.19 MDa at 47 °C), and HA production

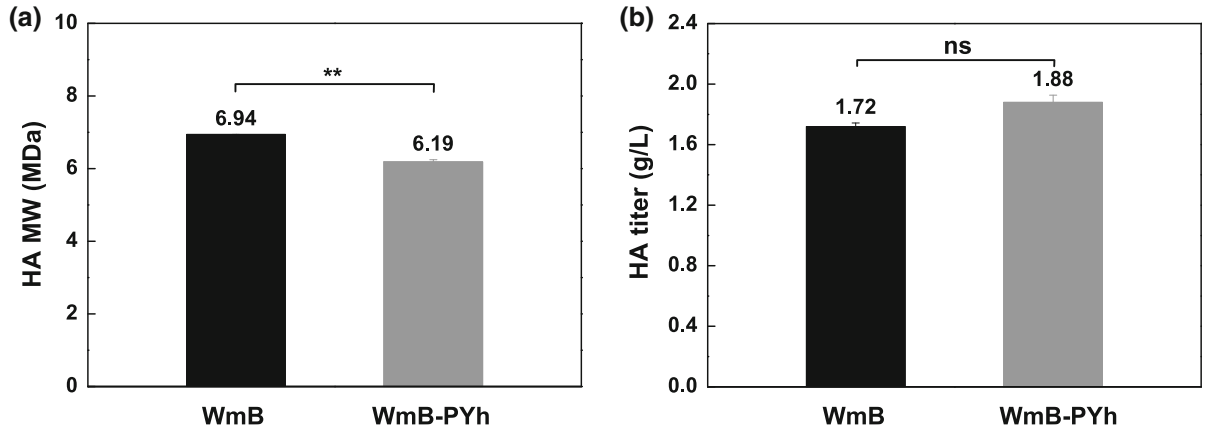

Fig. 4 HA MWs (**a**) and titers (**b**) for WmB and WmB-PYh at 47 °C. Data are averages of three independent experiments and error bars represent ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, no significance)

was efficient (4.25 g/L at 32 °C, 2.77 g/L at 42 °C and 1.88 g/L at 47 °C). To our knowledge, this is the first method for producing HAs with different MWs using a single bacterial strain. This strategy could also be employed to produce other valuable polysaccharides with different MWs.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval This work did not include any studies with human participants or animals.

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