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Expression of a novel hyaluronidase from *Streptococcus zooepidemicus* in *Escherichia coli* and its application for the preparation of HA oligosaccharides

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ABSTRACT

Hyaluronan (HA) oligosaccharides which can stimulate angiogenesis and suppress the growth of tumors have attracted more and more attention. In order to prepare pure and well-defined oligosaccharides from high-molecular-weight HA in a rapid and simple manner, an enzymatic degradation method was developed, which included degradation with a novel recombinant hyaluronan lyase (HA lyase, hyaluronidase, or HAase) and gel permeation chromatography. The HAase protein was expressed in *Escherichia coli* with the expression vector pBV220. The HAase was purified and refolded, and specific activity of the enzyme solution was 3800 U/mg. HA was degraded with HAase at the optimized conditions, yielding 46% and 31% of HA disaccharides and HA tetrasaccharides, respectively. These HA oligosaccharides were conveniently separated by consecutive column chromatography on Bio-gel P6 and were identified by HPLC–MS.

1. Introduction

Hyaluronan (HA) molecules are long, unbranched chains of variable length that consist of repeating disaccharide subunit of D-glucuronic acid (β1 → 3) and *N*-acetyl-D-glucosamine (β1 → 4) (Weissman & Meyer, 1954). Owing to its stable properties and high viscosity, HA and its derivatives are widely used in food, cosmetics, and pharmaceutical industries.

Bacterial HA lyase (hyaluronidase, HAase) degrades HA by cleaving *N*-acetylglucosamidic bonds of HA by a β-elimination process, producing HA oligosaccharides (o-HAs). The products are unsaturated oligosaccharides, often disaccharides, with a D-4, 5-uronate residue at nonreducing termini which has an optimal absorption at 232 nm (Yamagata, Saito, Habuchi, & Suzuki, 1968). HA oligosaccharides of defined length play a crucial role in dynamic cellular processes. For example, such HA preparations have been shown to promote angiogenesis (Rahmanian et al., 1997; West, Hampson, Arnold, & Kumar, 1985), induce expression of inflammatory mediators in alveolar macrophages (Horton, Shapiro, Bao, Lowenstein, & Noble, 1999; McKee et al., 1996; Noble, McKee, Cowman, & Shin, 1996) and inhibit tumor growth *in vivo* (Zeng, Toole, Kinney, Kuo, & Stamenkovic, 1998) as well as having many other effects (Fitzgerald, Bowie, Skeffington, & O'Neill, 2000; Oertli, Beck-Schimmer, Fan, & Wuthrich, 1998; Ohkawara et al., 2000).

HA oligosaccharides can be prepared conventionally by acid hydrolysis of HA. However, the hydrolytically active sites are on the C1, C4, and carbonyl carbons, and the hydrolysis product is not homogenous. Hydrolysis at C1 might involve two reaction processes, glycoside bond cleavage 1–3 and ring opening, while hydrolysis at C4 results in a one step reaction for glycoside bond cleavage (1–4) (Tokita & Okamoto, 1995). In addition, the hydrolyzing reaction is not easily controllable. Therefore, acid hydrolysis is not appropriate for large-scale preparation of HA oligosaccharides with high purity. Enzymatic degradation of polysaccharides should be a promising alternative to acid hydrolysis. The enzyme degrades polysaccharides with high specificity and under mild conditions.

HAase is present in a wide variety of bacteria, including several pathogens. Within the streptococci, HAases have been identified in Lancefield groups A, B, C, and G as well as *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus intermedius*, *Streptococcus consellatus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Berry et al., 1994; Calvinho, Almeida, & Oliver, 1998; Gunther, Ozegowski, & Kohler, 1996; Homer, Denbow, Whiley, & Beighton, 1993; Hynes & Walton, 2000; Schaufuss, Sting, Schaeg, & Blobel, 1989), but HAase of *Streptococcus zooepidemicus* has, as yet, not been reported. The role of HAase as a virulence factor in this bacteria is unclear.

In this study, a simple method of preparing diverse HA oligosaccharides was developed, in which high-molecular-weight HA was degraded by a recombinant HAase, HYL, yielding HA disaccharides (o-HA2), HA tetrasaccharides (o-HA4), HA hexa-saccharides (o-HA6), and HA 8 saccharides (o-HA8), which were then purified

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through gel-filtration chromatography and identified using HPLC–MS.

2. Experimental

2.1. Materials

The HAase gene was isolated from *S. zooepidemicus* cvcc 2336 (Veterinary Culture Collection Center of China). *Escherichia coli* DH5α (Life Technologies, Rockville, MD) was routinely used for transformation and propagation of plasmids. *E. coli* JM109 (Stratagene, La Jolla, CA) was used as the host strain for gene expression. pBV220 (Stratagene) was used to construct an expression vector. The pUC19 vector (Promega, Madison, WI) was used as a cloning vector and also as a sub-cloning vector for DNA sequencing. Ampicillin was purchased from Sigma, and sodium hyaluronate was obtained from Freda Biochem Co., Ltd. (Shandong, China). Protein molecular weight standards and DNA marker were purchased from Fermentas. All other chemicals were purchased from Sigma.

2.2. Cloning of HAase gene

Streptococcus zooepidemicus was cultured in TSB medium (Huang, Chen, & Chen, 2006) at 37 °C. Genomic DNA was extracted from cells using the CTAB protocol (Sambrook & Russell, 2001). The DNA concentration of each sample was determined by the absorption value at 260 nm. According to the HAase amino acid sequences of *S. agalactiae* (Accession No. AAN00079) and *S. pyogenes* (Accession No. AAF29533), a DNA fragment having high similarity with the two HAase genes was found in the genome of *Streptococcus equi*. Based on this DNA fragment, a pair of primers, HYL1-F [5′-CGGGATCCATTTAGAGGCTGTGTGCACC-3′] and HYL1-R [5′-ATACTGCAGAATGTGCTTGAATGGCTATG-3′], were designed for the PCR experiment. The product of the right size was purified by gel DNA extraction and then cloned into pUC19 vector. Subsequently, the ligated plasmid pUC19-HYL was sequenced to confirm that the desired product had been obtained.

2.3. Expression of HAase in Escherichia coli JM109

For expression of HAase ORF, a 2.6 kb DNA fragment encoding 862 amino acid was amplified by PCR from the plasmid pUC19-HYL and two synthetic primers, HYL2-F [5′-ATAGAATTCTTGT GGCTTCGATGGCAA-3′] and HYL2-R [5′-TAAGGATCCGCTTGAAT GGCTTGATAAG-3′], containing EcoRI (in the forward primer) and BamHI (in the reverse primer) restriction enzyme sites (underlined) under the following condition: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 6 min, and the final extension for 10 min. The PCR product was digested with EcoRI and BamHI restriction enzyme, and then ligated to EcoRI/BamHI digested pBV220 prokaryotic expression vector. The resulting recombinant plasmid (pBV220-HYL) was then confirmed by sequencing.

Expression of HAase fusion protein was carried out by the temperature induction (42 °C) after transformation of the recombinant expressing plasmid into *E. coli* JM109. The cells were cultured at 30 °C in 250 mL Luria–Bertani (LB) medium containing 100 μg/mL ampicillin. Cultures in log phase growth were induced at 42 °C and the cells were harvested after a further 4 h of cultivation by centrifugation at 8000*g* for 10 min. The pellet was washed twice with 50 mM acetic acid buffer (pH 6.0), and then resuspended thoroughly in the acetic acid buffer. The cell pellet was subjected to a sonication treatment in an ice-bath, and sonication was carried out at 300 W for 30 min in short bursts (sonication for 5 s and intermission for 5 s) in order to avoid overheating the mixture. The lysate was centrifuged at 10,000*g* for 15 min at 4 °C. The expression level was analyzed by gel analysis software after SDS–PAGE.

2.4. Preparation of HAase solution

Cells were lysed by sonication and centrifuged to isolate the HAase inclusion bodies. The inclusion body pellets were washed with 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM EDTA and 2% deoxycholate (Khan, AppaRao, Eshwari, Totey, & Panda, 1998). Subsequently, the inclusion bodies were washed with distilled water to remove contaminating salt and detergent and centrifuged at 8000*g* for 30 min. At this stage, the main component of the inclusion bodies was HAase. Then the purified HAase inclusion bodies were solubilized in 100 mM Tris–HCl buffer (pH 8.0) containing 8 M urea, 150 mM NaCl, 1 mM EDTA, and 0.5% β-mercaptoethanol for 12 h at 4 °C, and clarified by centrifugation at 12,000*g* for 30 min at 4 °C. The supernatant containing the solubilized IBs was collected and applied to a Sephadex G25 size-exclusion column equilibrated with buffer containing 2 M urea, 50 mM NaCl, and 50 mM Tris–HCl, pH 8.0. Then the column was eluted with the same buffer at a flow rate of 9 cm/h. The collected protein fractions were diluted to 0.1 mg/mL with redox refolding buffer containing 2 M urea, 50 mM NaCl, 50 mM Tris–HCl, pH 8.0, and 1 mM glutathione (0.1 mM glutathione oxidized and 0.9 mM glutathione reduced). The solution was stirred gently at 4 °C for 48 h to ensure the protein refolded. After refolding, the fusion protein solution was thoroughly dialyzed against water overnight at 4 °C and concentrated by ultrafiltration.

2.5. Preparation of HA oligosaccharides

HA (500 mg) was dissolved in 100 mL water, and then the solution was mixed with 0.3 mL HAase solution and incubated at 37 °C for 2 h. The hydrolytic reaction was stopped by heating the mixture in a boiling water bath for 20 min. After being cooled down to 4 °C, the hydrolyzate was centrifuged at 12,000*g* for 10 min, and then the supernatant was concentrated to 2 mL through rotary evaporation at 45 °C.

2.6. Purification of HA oligosaccharides

The HA degradation preparations (2 mL) were applied onto a Bio-gel P6 column (95 cm × 1.5 cm, Bio-Rad Laboratories, USA). NH_4HCO_3 (0.5 M) was used as eluent at a flow rate of 12 mL/h. The eluent from the column was detected by the Elson-Morgan method (Reissig, Strominger, & Leloir, 1955), and peaks, corresponding to different HA oligosaccharides, were identified by MS. Fractions (2 mL) were collected. The salt (NH_4HCO_3) was removed by repeated evaporation under diminished pressure at 60 °C. After a second column chromatography using the same conditions, the recovered HA oligosaccharides were lyophilized.

2.7. Analytical methods

2.7.1. Assay for protein concentration and molecular weight

The protein concentration was determined by BCA protein assay with bovine serum albumin as standard. SDS–PAGE analysis was carried out using a 12% polyacrylamide gel. After electrophoresis, protein was stained with Coomassie brilliant blue R-250.

2.7.2. Assay for HAase activity

To determine the HAase activity quantitatively, the enzyme source was incubated with 5 mg/mL HA aqueous solution in 50 mM acetic acid buffer (pH 6.0) at 37 °C. The degradation products were then determined as *N*-acetyl-glucosamine (NAG) equiv-

alents by the Elson-Morgan method. The rate of release of unsaturated oligosaccharides by the enzyme from HA, measured as the rate of increase in absorbance at 585 nm, was measured by using a Unic UV-2100 spectrophotometer. The amount of the HAase that releases 1 μmol of the unsaturated oligosaccharides product per minute at 37 °C was considered as one enzyme unit.

2.7.3. HPLC–MS analysis of HA oligosaccharides

HPLC–MS was conducted on a Waters 1525 HPLC (Waters, Milford, USA), coupled with an ESI detector (Waters Micromass ZQ2000, Waters, Milford, USA). Anion exchange chromatography-HPLC equipped with YMC NH2 column (4 × 250 mm) was performed using acetonitrile solution (90:10, v:v) under constant flow (1 mL/min) at 35 °C. The eluent was monitored at 210 nm. Mass spectra in the negative-ion mode were generated under the following conditions: fragmenter voltage =100 V; voltage = 2800 V; nebulizer pressure = 25 psi; temperature = 200 °C; m/z range = 50–2000.

3. Results and discussion

3.1. Characterization of HAase gene

The HAase encoding gene was amplified using the total DNA as the template and ligated to pUC19 vector to construct a sub-clone vector. The products of PCR were separated by 1.5% agarose gel electrophoresis. It was shown that there was a specific band at about 2.7 kb, which was in accordance with expectation. The analysis of this sequence revealed an open reading frame (ORF) likely to be that of *hyl* gene. This ORF starts with ATG at position 167 and terminates with TAG at position 2652, encoding a putative protein of 862 amino acid residues. At 6 bases upstream from the ATG initiator codon, there is a putative ribosome-binding site, which consists of 10 bases sequence, AACGCTTGTG (Fig. 1). The DNA sequences obtained in this study were submitted to GenBank as EU082206 for *S. zooepidemicus*.

The deduced amino acid sequence of HYL from *S. zooepidemicus* has a high degree of homology to other members of the glycosaminoglycan (GAG) polysaccharide lyase (Fig. 2). The predicted amino acid sequence of HYL showed 48%, 43%, and 42% identity to that of *S. agalactiae* 2603V/R (AAN00079) HAase, *S. pneumoniae* D39 HA lyase (ABJ54273), and *S. pyogenes* HylA (AAF29533), respectively. As expected, the active sites (N303, H364, and Y373) of HYL from *S. zooepidemicus* were highly conserved (Fig. 2). It was inferred that the *hyl* gene absolutely existed in the genome of *S. zooepidemicus*, but this strain was still able to produce high-molecular-weight HA. The reason was probably that the space between the putative −10 and −35 sequence was so long (37 bases) that HAase was not expressed during the process of HA production.

3.2. Expression and purification of HAase in E. coli JM109

Although the expression level of some plasmid vectors such as pET-28b, pET-22b, and pET-42b in which the expression is induced chemically is higher than that of pBV220, we used plasmid pBV220 to construct the expression vector in our experiments. The main reason is that these vectors are only good for the expression of recombinant protein on a small laboratory-scale rather than for a large scale of expression. Plasmid pBV220 is a prokaryotic high-level expression vector and is constructed by Zhang, Yao, and Hou (1990). It contains bacteriophage λ P_RP_L promoters and cIts857 allele, multiple cloning sites (MCS), and two strong transcription terminators. Therefore, it can be transformed into any *E. coli* strains. Referring to the literature, pBV220 vector affords a high-level expression induced by temperature, which has been proved in our experiments. Furthermore, compared with the expression system induced by IPTG, the temperature-inducible system is not toxic to *E. coli*. Therefore, the transformed *E. coli* can grow well and the expressive level can reach a high level eventually.

DNA fragments with the complete ORF were inserted into pBV220, resulting in pBV220-HYL. Plasmid pBV220-HYL was transferred into *E. coli* JM109. The transformant was designated JMHYL. After induction at 42 °C for 4 h, SDS–PAGE image analysis showed that the JMHYL cells produced a large amount of a new protein (molecular weight approximately 96 kDa) (Fig. 3). More than 80% of the over-expressed protein existed as insoluble form. Compared

```
att tag agg ctg tgt gca cca aac cgt tgc cat caa tcc aga caa   45

gca gta ttt gct gac ctt tga cat tga gac aaa gga taa aac agg   90
                        -35

tca ggc ttt tgc gcg tat cat tga aga aat aaa gca agg ttc tgg   135
                      -10

tgc tgc aaa aga gca acg ctt gtg gct tcg atg gca aca gga act   180
                      RBS               Met Ala Thr Gly Thr   60

gag aaa aaa cac caa gaa aag ctt tat atc cct aag cta aag gtt   225
Glu Lys Lys His Gln Glu Lys Leu Tyr Ile Pro Lys Leu Lys Val   75

------------------------------------------------------------

tat caa aag gtg gga aat gac ttt aag cag cta tct ttt aag gcc   2745
Tyr Gln Lys Val Gly Asn Asp Phe Lys Gln Leu Ser Phe Lys Ala   915

tta tca tag cca ttc aag cac att   2769
Leu Ser End
```

Fig. 1. The nucleotide sequence of flanking regions of *hyl* gene. The −35 and −10 regions of a putative promoter sequence and a putative ribosome-binding site are underlined.

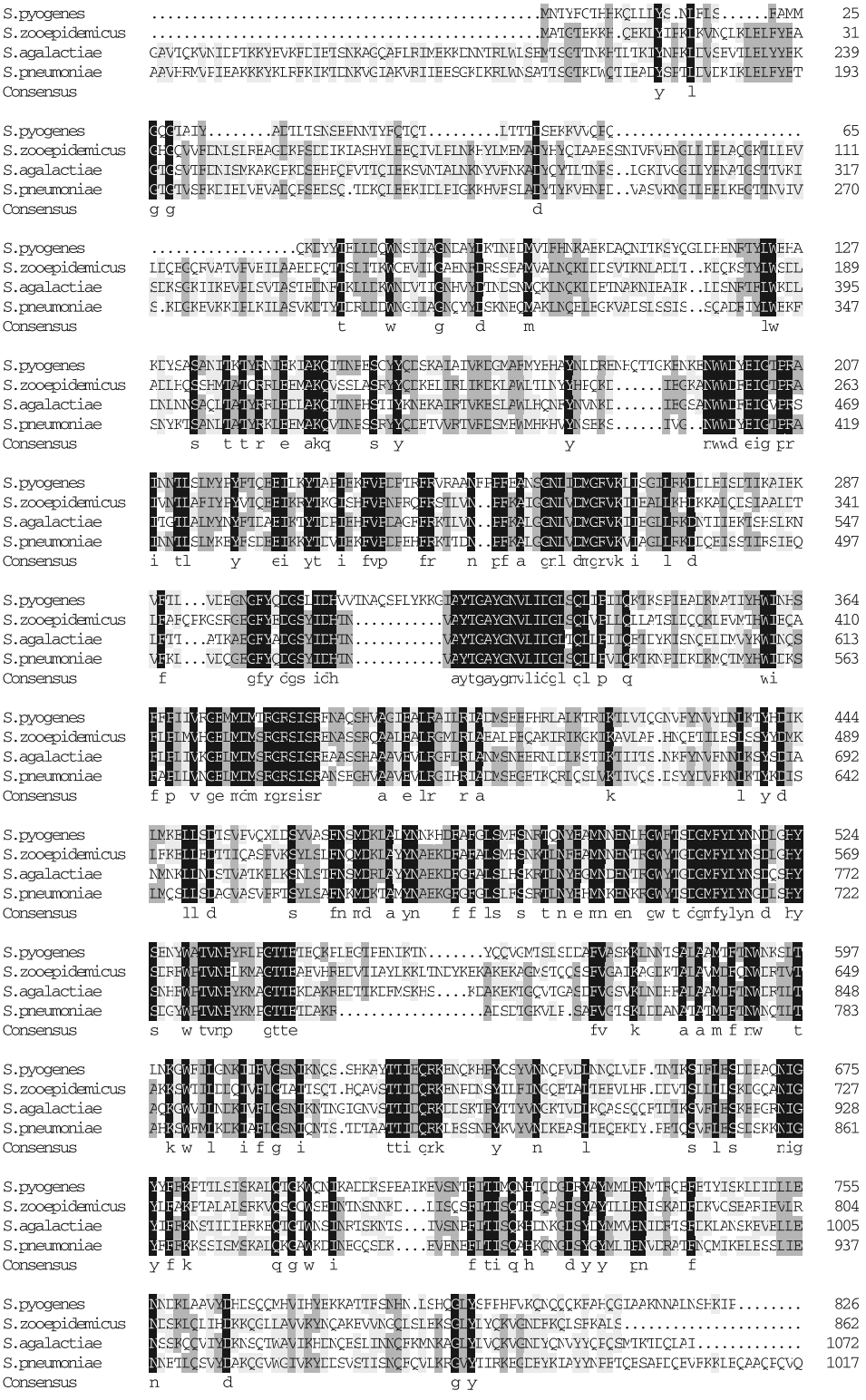

Fig. 2. Alignment of the deduced *S. zooepidemicus* HYL amino acid sequence with Glycosaminoglycan polysaccharide lyase family enzymes, *S. agalactiae* 2603V/R hyaluronidase, *S. pneumoniae* D39 HA lyase, and *S. pyogenes* HylA. Black boxes, identical amino acids between *S. zooepidemicus* HYL and other enzymes.

with the molecular mass of other bacterial HAases (77–121 kDa) (Hynes & Walton, 2000), the molecular mass of HYL was moderate.

The HYL protein was refolded and purified on a Sephadex G25 size-exclusion column. After purification, the purity was above 95% and the concentration was about 1.2 g/L detected by BCA method. The catalytic degradation activity was tested using both the suspension of the sonication lysate and the refolded HYL (Table 1.) The results suggested that the suspension of the sonication lysate contained some active HAase and *E. coli* JMHYL was able to express some soluble HAase besides the inclusion bodies. Significant degradation of HA was seen during 2–3 h of reaction, which was represented by the viscosity of the HA solution, the contents of

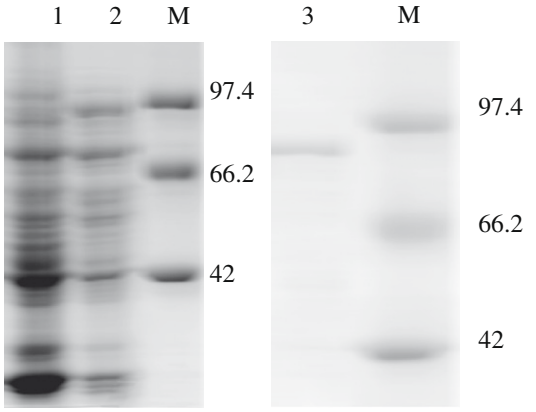

Fig. 3. Samples from HYL purification steps. Proteins were separated by SDS–PAGE (12%) and visualized by Coomassie brilliant blue G-250 staining. Lane M, molecular mass standards; lane 1, lysate from *E. coli* JMN cells 4 h after induction; lane 2, lysate from *E. coli* JMHYL cells 4 h after induction; lane 3, refolded protein HYL.

Table 1
Enzyme activity of cell lysate or refolded protein.

Escherichia coli strain or refolded protein	Plasmid	HYL activity (U/mg)
JM109 (host)		0
JMHYL	pBV220-HYL	3100
JMN	pBV220	0
Refolded HYL		3800

Escherichia coli JMN was developed by transferring the plasmid pBV220 into *E. coli* JM109. *E. coli* JMHYL contained the transferred plasmid pBV220-HYL. Refolded HYL was the renaturated HYL followed by purification on a Sephadex G25 size-exclusion column. Values are means of three replicates.

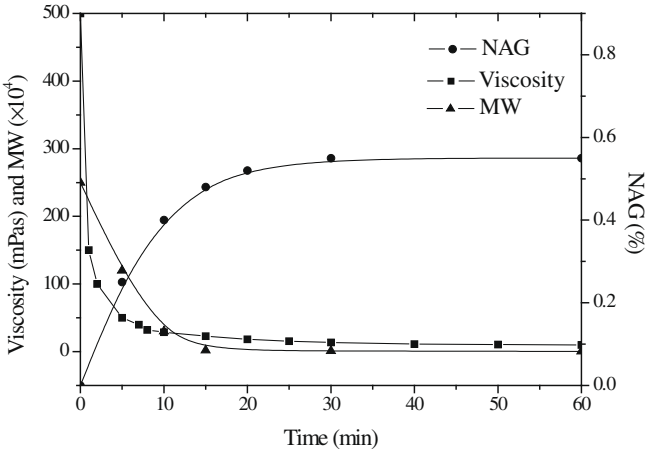

Fig. 4. The viscosity of HA solution, NAG content and molecular weight (MW) of HA during degradation treatment. (■) The viscosity of HA solution; (●) NAG content; (▲) MW of HA.

the NAG and the molecular weight of HA (Fig. 4). During degradation, the viscosity and the molecular weight of the HA solution reduced rapidly and the contents of the NAG increased. Compared with the specific activity of HAase of *S. pneumoniae* and bacteriophage H4489A expressed in *E. coli* (Li et al., 2007; Jedrzejas, Mewbourne, Chantalat, & McPherson, 1998), the specific activity of purified HYL was about 1.5-fold lower than that of *S. pneumoniae* HAase, but 400-fold higher than that of bacteriophage H4489A HAase. Temperature and pH optimum studies are presented in Fig. 5. The results showed that the optimum reaction temperature was 37 °C, while the highest reaction activity appeared at pH 6.0.

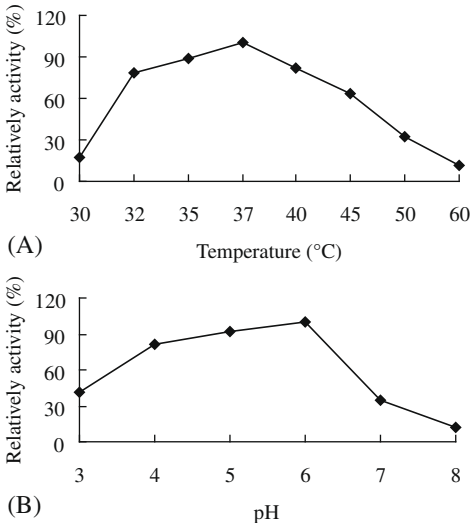

Fig. 5. Temperature and pH dependency of HYL. (A) Temperature; (B) pH.

Besides, the purified HYL was quite stable since its activity declined 20% after 5 days incubation at room temperature.

HAase of *S. zooepidemicus*, unlike most bacterial HAases, was found to be exclusively specific for HA. By Elson-Morgan method, HAase activity was detected in the cell lysate of *E. coli* JMHYL carrying *hyl* gene. The sample from *E. coli* JMN carrying only a vector had no enzymatic activity. When using CS-A as substrate, although the cell lysate of JMHYL carrying the *hyl* gene had activity to degrade HA, it did not show CS-A depolymerization activity. This enzymatic property was the same as that in the culture supernatant of *S. pyrogens* bacteriophage H4489A (Baker, Dong, & Pritchard, 2002).

3.3. Preparation and analysis of HA oligosaccharides degraded by HYL

It is important that well-characterized and highly purified o-HA is used in biological and structural studies, so we established a method for the purification of o-HA of defined molecular weight. The optimal reaction conditions of HA hydrolysis catalyzed by HYL were studied by orthogonal design (Tables 2 and 3). It was found that the hydrolysis reaction was affected by temperature, pH, substrate concentration, the amount of HYL used and reaction time. The optimal reaction condition was as follows: 0.5% of HA, 1.5×10^4 U/L of HYL, pH 6.0, reaction temperature 37 °C, reaction time 2 h. Most of HA was degraded into oligosaccharides under the above condition. The remaining HA was removed easily with centrifugation because the polymer would form white flocculent precipitation with the protein when heated. The supernatant of HA degradation was concentrated by rotary evaporation and then purified with a chromatography column.

Three chromatography columns were selected to study their abilities of separating o-HAs. HA degradation samples (2 mL) were

Table 2
Level of experiment factors.

Factor level	A HA concentration (%)	B pH	C *t* (h)	D HYL concentration (U/L)	E *T* (°C)
1	0.25	5.0	0.5	0.5	35
2	0.50	6.0	1.0	1.0	37
3	0.75	7.0	1.5	1.5	39
4	1.00	8.0	2.0	2.0	41

Table 3
Results of orthogonal design.

	A	B	C	D	E	Degradation efficiency (%)
1	1	1	1	1	1	64.70
2	1	2	2	2	2	71.00
3	1	3	3	3	3	77.34
4	1	4	4	4	4	40.84
5	2	1	2	3	4	80.64
6	2	2	1	4	3	84.70
7	2	3	4	1	2	60.86
8	2	4	3	2	1	29.08
9	3	1	3	4	2	68.76
10	3	2	4	3	1	76.78
11	3	3	1	2	4	23.70
12	3	4	2	1	3	15.98
13	4	1	4	2	3	35.14
14	4	2	3	1	4	31.48
15	4	3	2	4	1	25.00
16	4	4	1	3	2	19.66
K_1	253.88	249.24	192.76	173.02	195.56	
K_2	255.28	263.96	192.62	158.92	220.28	
K_3	185.22	186.90	206.66	254.42	213.16	
K_4	111.28	105.56	213.62	219.30	176.66	
R	36.00	39.60	5.25	23.87	10.91	

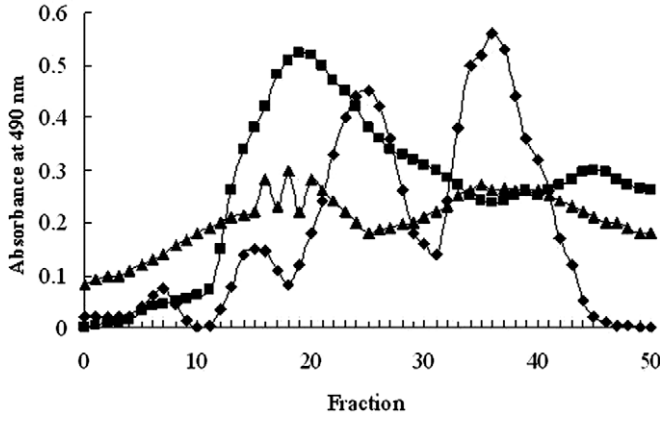

Fig. 6. Gel-filtration on Sephadex G25, Bio-gel P6, and Dowex 1 × 2 of the HA oligosaccharides derived from HA degradation. (♦) Bio-gel P6; (■) Sephadex G25; (▲) Dowex 1 × 2.

applied on the columns of Sephadex G25, Bio-gel P6, and Dowex 1 × 2, respectively. As shown in Fig. 6, o-HAs were better separated using Bio-gel P6 column than the other two columns. These o-HAs were eluted as four peaks from a Bio-gel P6 column. The products of HA degradation were mainly o-HA2 (46%) and o-HA4 (31%) with a small amount of o-HA6 (16%), and o-HA8 (7%), which was confirmed by HPLC–MS. They were collected and concentrated separately and purified again with Bio-gel P6 column. More than 90% of the oligosaccharides were recovered after two chromatography steps. After the second gel-filtration, the purity of the HA oligosaccharides reached 99%. In order to determine whether Bio-gel P6 column chromatography can be used for the preparation of HA oligosaccharides on a large scale or not, the HAase hydrolyzate of 5 g HA was applied on an enlarged P6 column (95 cm × 4.5 cm). It was found that this column could purify diverse oligosaccharides with similar recovery rates and resolutions comparable to the smaller column. HPLC–MS spectroscopy was used for the structure determination of the different oligosaccharides. HPLC–MS confirmed the molecular weights of 379, 758, 1137, and 1516 Da for o-HA2, o-HA4, o-HA6, and o-HA8, respectively.

The results indicate that HA is specifically depolymerized by HYL under mild condition and the molecular weight of o-HAs obtained are homogenous. Every o-HA have only one structure. However, o-HAs obtained by acid hydrolysis method are not homogenous. The glycoside ring may be destroyed, and one kind of o-HA may have different structures due to the three different hydrolytic active sites (C1, C4, and carbonyl carbons). Further more, acid hydrolysis process will produce many monosaccharides, and the waste acid solution from the process can lead pollution to environment. Therefore, enzymatic degradation of HA is a better method compared with acid hydrolysis method.

The results also confirm that the HAase of *S. zooepidemicus* has different degradation mechanism from mammalian HAase. While HA degradation by streptococcal HAase involves an initial random endolytic cleavage followed by rapid exolytic and processive release of unsaturated oligosaccharide (Li & Jedrzejas, 2001), the bovine HAase acts as an endolytic enzyme with tetra- and hexasaccharides as the main degradation products (Cramer, Bailey, Bailey, & Miller, 1994). The HA oligosaccharides obtained by streptococcal HAase degradation have obvious UV absorption at 232 nm, which can be used to distinguish between unsaturated oligosaccharides and saturated oligosaccharides.

In conclusion, HAase from *S. zooepidemicus* has been expressed successfully in *E. coli* as non-fusion proteins and the purification of recombinant protein is relatively simple and effective. The new HAase is highly active and specific. The whole process is easy to handle and particularly it can be used for the large-scale expression of the objective proteins in a convenient manner. Furthermore, Bio-gel P6 gel-filtration can separate HA oligosaccharides with a good resolution. These advantages allow us to successfully prepare four HA oligosaccharides. The entire procedure including pre-cultivation of the HAase producing strain can be completed in one week. The real working time is less than 30 h, since most of the steps, such as degradation, chromatography, and lyophilization, do not require constant attention. Preparative scales over 25 mg can be easily reached in one experiment and each step of the method can be scaled up to some degree. The establishment of this simple and fast method will open a new avenue for the research on structure–function relationship of HA oligosaccharides and their industrial preparation.

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