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Cloning of Hyaluronan Synthase (sz-*has*) Gene from *Streptococcus zooepidemicus* in *Escherichia coli*

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ABSTRACT: A 546-bp fragment of the *sz-has*A gene encoding hyaluronan synthase (szHAS) from *Streptococcus zooepidemicus* (group C Streptococcus, GCS) was amplified by PCR with oligonucleotides designed based on the conserved amino acid sequences of HASs from other organisms as primers. The entire *sz-has*A gene was identified and cloned by Southern and colony hybridizations using this 546-bp fragment as a probe. Determination of the nucleotide sequence indicated that this gene encoded a protein with 417 amino acid residues (calculated molecular mass, 47.77 kDa). The amino acid sequence of szHAS was 74.2% identical to that of HAS from *Strep. equisimilis*. The overexpression of *sz-has*A in *Escherichia coli* was detected by SDS-PAGE and confirmed by LC/MS-MS. To examine whether *E. coli* cells acquire an ability to synthesize hyaluronic acid (HA) upon transformation with plasmids bearing the *sz-has*A gene, the plasmids pHAS-1 and pHAS-2, in which *sz-has*A and *sz-has*A with rare codon modifications at the 5'-terminus were ligated into the *Nde*I-*Bam*HI sites of pET-28a, respectively, were constructed and used to transform *E. coli* BL21 (DE3), *E. coli* BL21 (codon+), and *E. coli* HMS 174 (DE3) plysS. Cultivation of these transformants, followed by induction for gene expression, revealed that the *E. coli* BL21 (codon+) transformants with pHAS-1 and *E. coli* HMS 174 (DE3) plysS transformants with pHAS-2 produced HA after 4 hr induction time at the maximum yield 16 μg/ml and 32.5 μg/ml, respectively. These are higher than the background levels in control bacteria, which were 5 μg/ml and 21.3 μg/ml, respectively.

KEYWORDS: Hyaluronic acid, Hyaluronan synthase, *Streptococcus* sp., Gene cloning.

INTRODUCTION

Hyaluronic acid or hyaluronan (HA) is a naturally occurring polysaccharide composed of alternating a β-1,4-glucuronic acid (GlcA) and β-1,3-*N*-acetylglucosamine (GlcNAc) moieties. HA is a major constituent of the vitreous humor of the eye, synovial fluid, extracellular matrices, and skin.[1] The polysaccharide also interacts with various receptors and binding proteins that modulate cellular behavior such as migration, adhesion, and wound healing.[1,2,3] HA is used in many skin care products because it is an excellent moisturizer. Principle uses of HA are in the biomedical field, such as in ophthalmic and orthopaedic surgery.[4,5] Initially, HA was mainly extracted from rooster combs, a specialized piece of skin which has a higher amount of HA, but the extraction and purification procedure are time consuming and labor intentive, making HA production very costly.[6] Alternatively, HA is produced by *Streptococcus zooepidemicus* through fermentation[7], by which the acid has been more conveniently obtained for industrial production. The equivalence of streptococcal and rooster comb HA has been demonstrated, and the former has now been accepted from a regulatory point of view. Supplies of HA from streptococcal fermentation are theoretically limitless, and no fears of seasonal fluctuations or batch-to-batch variation exist if a tightly controlled process is implemented.[5] The HA is produced as a capsule of high-molecular weight material which is easily separated from the microorganisms, although extensive purification is still necessary in order to obtain material of sufficient purity. The possibility of contamination with viral agents is obviously not a major concern, and the use of non-pathogenic mutant in the industrial processes will ensure the absence of other toxic impurities.[8]

Therefore the purpose of this paper is to use recombinant DNA technology to improve HA production from *E. coli*. In the work reported here, we cloned and expressed *Strep. zooepidemicus* hyaluronan synthase gene (*sz-has*A) in *E. coli* cells and investigated the productivity of HA from the recombinant HA-producing strains.

Materials and Methods

Materials

Media were purchased from Difco (Michigan, USA). Restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, WI, USA). Synthetic oligonucleotides were made at the Bioservice Unit, National Science and Technology Development Agency, Bangkok, Thailand. All other reagents were of the highest grade available from Sigma-Aldrich company (St. Louis, MO, USA) except stated otherwise.

Microorganisms and plasmids

The selected strain of *Streptococcus zooepidemicus* ATCC 35246 was used throughout the experiments. The *Escherichia coli* host strain TOP 10 was used as a host for pCR[a]-Blunt and pBAD-TOPO[a] vector (Invitrogen, Carlsbad, CA, USA). *E. coli* strain JM 109 was used as a host for pUC18/19, PCR 2.1 cloning vector (original TA cloning vector kit) (Novagen, Madison, WI, USA) and pCR[a]- Blunt cloning vector. *E. coli* strain BL21 (DE3), strain BL21 (codon+) and strain HMS 174 (DE3) pLysS were used as a host for pET-28a expression vector (Novagen). *Strep. zooepidemicus* was grown in Brain Heart Infusion (BHI) medium and *E.coli* strains were grown in LB medium.

Isolation of streptococcal DNA

Genomic DNA was isolated from *Strep. zooepidemicus* by the method of Caparon and Scott (1991).[9] Briefly, 100 ml of overnight culture was centrifuged at 10,000 x g for 10 min at 4 °C and the pellet was washed with 10 ml of Tris buffer (20 mM Tris-HCl, pH 8.2). Bacterial cell walls were disrupted by resuspension in 3.2 ml of the same Tris buffer, then 7 ml of polyethylene glycol solution (PEG 20,000, Fisher Scientific, Fairlawn, NJ, USA, 24%, w/v, in water, sterilized by filtration) and 3.5 ml of lysozyme solution (Sigma L-6876, 20 mg/ml in water) were added. The mixture was incubated for 1 hr at 37°C and was subsequently centrifuged (15,000 x g, 10 min, 4 °C). The pellet was resuspended in 5.7 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated with 0.3 ml of a 20% (w/v) aqueous solution of SDS for 15 min at 65 °C. RNase A solution (Sigma R-4875, 5 mg/ml in water) was added and incubated for 30 min at 37 °C with 0.2 ml of proteinase K solution (Sigma P-0390, 10 mg/ml in water) and then incubation was continued for 30 min at 37 °C. The lysate was then extracted once with TE-saturated phenol, once with phenol-chloroform (1:1) and once with chloroform. The residual chloroform was evaporated and 1/10 the aqueous volume of 3 M sodium acetate and 2 volumes of 95% ethanol were subsequently added. The DNA was collected by centrifugation (5000 x g, 4 °C, 5 min) and washed twice with 5 ml of 70% (v/v) ethanol. The pellet was dried under reduced pressure and resuspended in 1 ml of TE.

Cloning of the sz- hasA gene

A part of the *sz-has*A gene was amplified by PCR using forward (5' TGG GGC GGC AAA CGT GAG GTC ATG TAC ACA GC 3') and reverse (5' CAC CAC AGA GCA TTG TAT AGC CAC TCT CGG AAG TA 3') primers. The genomic DNA of *Strep. zooepidemicus* was used as a template, PCR was performed with *Taq* DNA polymerase (Promega, USA) in a thermocycler (Geneamp PCR System 2400, Perkin Elmer, Foster-city, CA, USA) using the following program: i) 1 cycle of 95°C for 2 min, 45°C for 3 min, and 72°C for 5 min; ii) 30 cycles of 95°C for 45 sec , 45°C for 30 sec, and 72°C for 1 min 30 sec. The resultant 546-bp fragment was separated on an agarose gel electrophoresis and purified using the Gene clean kit (BIO 101. Inc., Vista, CA, USA). The entire *sz-has*A gene was identified by Southern and colony hybridizations using this DNA fragment as a probe against chromosomal DNA of *Strep. zooepidemicus*. The chromosomal DNA was digested with *Hin*dIII, *Bam*HI, *Eco*RI, *Sma*I, *Sal*I or *Sac*I. Determination of the nucleotide sequence of the 1.5-kb *Sac*I fragment revealed that it contained the entire *sz-has*A gene. Southern hybridization was performed according to standard procedures. Probe labelling, hybridization and immunological detection were performed using the digoxigenin DNA labelling system (Boehringer Mannheim GmbH, Mannheim, Germany). The DNA fragments which gave the strongest signal, were extracted from a preparative agarose gel and ligated into the appropriate site of the pUC19 vector to generate a plasmid library. The *E. coli* JM109 cells were transformed with this plasmid library and screened for those containing the *sz-has*A gene by colony hybridization.

DNA sequence analysis

DNA sequencing of both strands of the insert was performed by the dideoxynucleotide chain termination method modified from that described by Sanger *et al*. (1977)[10] with fluorescent vector primers (Auto Read Sequencing kit, Pharmacia Biochemicals, Uppsala, Sweden) using an automated DNA sequencing system (ALF express, Pharmacia Biochemicals, Uppsala, Sweden). The nucleotide and deduced amino acid sequences were analyzed using DNASIS™ software (Hitachi software Co. Ltd., Tokyo, Japan).

Subcloning sz-hasA gene into expression vector

A full-length 1.4–kb *sz-has*A gene was amplified with *Taq* DNA polymerase (Promega, USA) from a positive clone obtained by colony hybridization as described above. The PCR primers (primer A and primer

B) were designed to contain *Nde*I-*Bam*HI sites (in italics) located at the start and stop codon regions of the *sz-has*A gene.

Nde I

primer A : (5'- GC *CAT ATG* AGA ACA TTA AAA AAC CTC ATA ACT G -3')

*Bam*HI

primer B : (5' – TC *GGA TCC* TTA TAA TAA TTT TTT ACG TGT TCC -3')

The PCR product was purified by agarose gel electrophoresis and cloned into the PCR 2.1 vector and then subcloned into pET-28a®(Invitrogen, USA), an expression plasmid, at the *Nde*I-*Bam*HI sites to obtain plasmid pHAS-1. Plasmid pHAS-2, which carries the *sz-has*A gene with rare codon modifications, was similarly constructed. The gene with rare codon modifications was constructed by PCR using the 5'-primer C, which was designed such that the codons for Arg, Thr and Ile (at amino acid position 2, 3 and 8, respectively) were changed from AGA to CGT, ACA to ACC and ATA to ATC (underlined), respectively.

Nde I Arg Thr Ile

primer C: (5'-GC*CATATG***CGTACC**TTAAAAAACCTC**ATC** ACTG-3')

Then nucleotide sequencing of the *sz has*A gene in the plasmids, pHAS-1 and pHAS-2, was performed.

Overexpression of szhasA gene in *E. coli*

Plasmids HAS-1 and HAS-2 were used to transform *E. coli* JM 109, *E. coli* BL21 (DE 3), *E. coli* BL21 (codon$^+$) and *E. coli* HMS 174 (DE3) pLysS. The transformants were grown at 37°C in LB medium containing kanamycin (50 µg/ml) until the optical density at 660 nm reached 0.4. Expression was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were incubated for 4 hr at 37°C. The cells were harvested by centrifugation 8,000x *g* for 10 min at 4°C and washed with buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA and 5 mM 2- mercaptoethanol). This step was repeated twice. The production of hyaluronan synthase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which was performed according to the method of Sambrook.[11]

Confirmation of production of recombinant szHAS in transformed *E. coli* by LC/MS-MS analysis

LC/MS-MS sample was prepared by neatly cutting the target band from SDS-PAGE. The reduction of disulfide bonds was subsequently performed by adding 10 mM DTT in 25 mM NH_4HCO_3 solution to the dried gel band. Tryptic digestion and extraction of peptides were then performed. LC/MS-MS analysis of the sample was done using a Finnigan LTQ Linear Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA) by the Bioservice unit, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand. The spray needle voltage was set at 1.8 KV and capillary temperature at 260 °C. The Turbo SEQUEST algorithm in the BioWorks ™ 3.1 SR1 software package (thermo Electron) and nr Fasta database were used for data analysis. The identified peptide were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides was Xcorr>1.5 for singly charged ions, Xcorr>2.0 for doubly charged ions, and Xcorr >2.5 for triply charged ions.

Determination of hyaluronic acid in culture supernatant

The hyaluronic acid concentration in the culture broth was determined by the carbazole method measuring uronic acid.[12,13,14] The culture broth was centrifuged at 3,000x g for 20 min. After the cells were removed, 2 volumes of ethanol were added to 1 volume of the supernatant from the culture broth and the solution was refrigerated at 4°C for 1 hr to precipitate the hyaluronic acid. The precipitate was recovered and redissolved in 9% NaCl. The assay detects the glucuronic acid released when the sample is hydrolyzed with H_2SO_4. This assay is not highly specific (compounds such as sucrose are cross-reactive) and so it is necessary to determine the background reading of the control bacteria. It was also confirmed that the colour reaction was specifically due to hyaluronic acid by the addition of hyaluronidase (Sigma-Aldrich company, St. Louis, MO), an enzyme specific for hyaluronic acid.

Nucleotide sequence accession number

The *Strep. zooepidemicus has*A nucleotide sequence has been assigned Accession No. AF 414053 by GenBank.

Results and Discussion

Cloning of the sz-hasA gene

Comparative analysis of the amino acid sequences of spHAS (*Strep. pyogenes*), DG42 (*Xenopus laevis* HAS)[15], nodC (a *Rhizobium meliloti* modulation factor)[16] and seHAS (*Strep. equisimilis*)[17] revealed the existence of the conserved regions WGGKREVMYT and YFREWLYNALWW. Therefore, we designed two deoxyoligonucleotide primers for PCR. The 546-bp fragment, which was amplified by PCR using these primers, was isolated, introduced into the pCR®-Blunt cloning vector, and its nucleotide sequence was determined. The deduced amino acid sequence showed high similarity to other known HAS sequences. In order to clone the entire *sz-has*A gene, Southern

hybridization was performed using the 546-bp fragment as a probe against chromosomal DNA of *Strep. zooepidemicus* which was digested with *Hin*dIII, *Bam*HI, *Eco*RI, *Sma*I, *Sal*I or *Sac*I. Determination of the nucleotide sequence of the 1.5-kb *Sac*I fragment revealed that it contained the entire *sz-has*A gene.

Sequence analysis of szHAS

The nucleotide sequence and the deduced amino acid sequence of szHAS, the enzyme that polymerizes HA from *Strep. zooepidemicus*, are shown in Fig. 1. The protein is composed of 417 amino acid residues with a calculated molecular mass of 47.77 kDa, and the predicted pI of 9.01. We found that its amino acid sequence was identical to that of HAS of *Strep. equisimilis* (seHAS), but the codon usages for 15 amino acids are different. It also showed high amino acid sequence

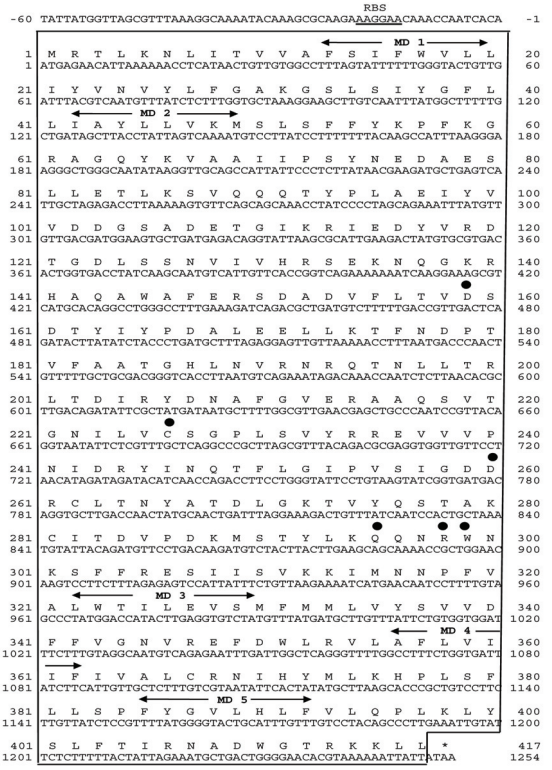

Fig 1. Nucleotide and deduced amino acid sequences of the *Strep. zooepidemicus* HAS ORF. The DNA sequence (bottom row) of the ORF encoding szHAS shown inside the rectangle was cloned from genomic DNA of *Strep. zooepidemicus*. The encoded amino acid sequence is shown on the top of the nucleotide sequence. The amino acids indicated by dots are conserved in all members of the β-glycosyltransferase family.[16] The approximate midpoints of predicted membrane domains (MD) 1 though 5 are indicated in arrows (using the algorithm of Kyte and Doolittle[24]). The hypothetical ribosome-binding site is *underlined* and labeled as *RBS*. The stop codon for *has*A is indicated by an *asterisk*.

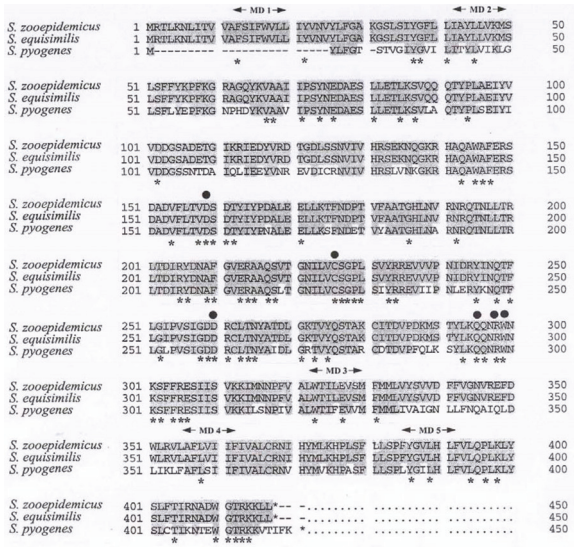

Fig 2. Alignment of the deduced amino acid sequence of HAS from *Strep. zooepidemicus* with *Strep. equisimilis* and *Strep. pyogenes*. The alignment was adjusted to the degree of overall sequence similarity with *Strep. zooepidemicus* using the DNASIS software (Hitachi Software, Yokohama, Japan). Identical amino acids are shown on a black background. Residues in szHAS that are identical in all other HAS family members (including human HAS1, HAS2 and HAS3, which are not shown) are denoted by asterisks. The amino acids indicated by dots are conserved in all members of the larger β-glycosyltransferase family[16]. The approximate midpoints of predicted membrane domains (MD) 1 though 5 are indicated.

identity of 74.2% to spHAS (Fig. 2).

The overall membrane topology predicted for szHAS (Fig. 3) was identical to that for seHAS, spHAS and the eukaryotic HASs reported thus far.[18] The protein has two putative transmembrane domains at the amino terminus and 2-3 membrane-associated or transmembrane domains at the carboxyl end. The hydropathy plots for the three streptococcal enzymes are virtually identical.

Subcloning sz-hasA gene into expression vector

When the expression of *sz-hasA* from various *E. coli* transformants with pHAS-1 were examined by SDS-PAGE, only *E. coli* BL21 (codon+) transformants were shown to express sz-HAS enzyme (Fig. 4). LC/MS-MS amino acid sequencing of the overexpressed band on SDS-PAGE confirmed it was sz-HAS protein (Fig. 5). HA synthase is a key enzyme in the biosynthetic pathway of HA.[8] Therefore, we expected that the recombinant sz-HAS expressed in *E. coli* BL21 (codon+) cell could synthesize HA. Determination of HA in culture supernatant showed that the strain could produce HA

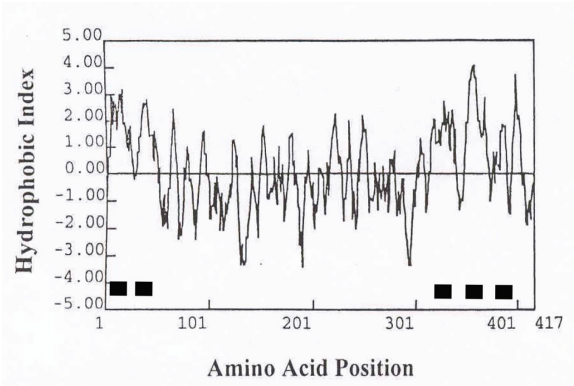

Fig 3. Hydropathic analysis of the deduced amino acid sequence of HAS from *Strep. zooepidemicus*. The hydropathic indices are on the Y-axis and amino acid numbers of HAS (1-417) are on the X-axis. The hydrophobicity values were obtained according to the algorithm of Kyte and Doolittle.[24] Positive values represent increased hydrophobicity. The predicted membrane-associated domains are marked with solid bars. The protein has two putative transmembrane domains at the amino terminus and 2-3 membrane-associated or transmembrane domains at the carboxyl end.

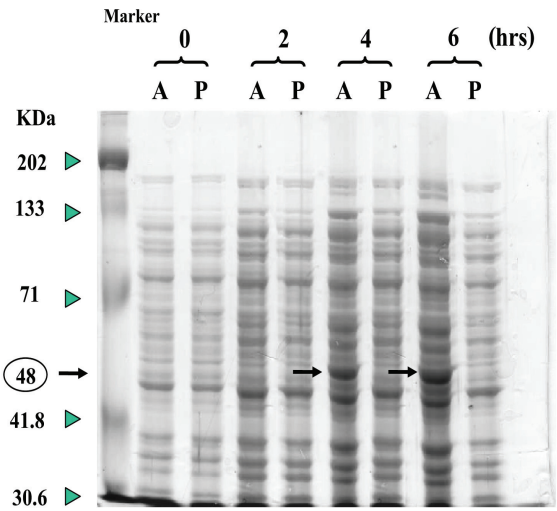

Fig 4. SDS-PAGE analysis of overexpressed streptococcal HAS in *E. coli* HMS 174(DE3) pLysS at induction time 0, 2, 4 and 6 hr. The 0.1% SDS-12% polyacrylamide gel was stained with Coomassie Blue R-250. The position of HAS is marked by the arrow.
lane A : *E. coli* HMS 174(DE3) pLysS containing pHAS-2.
lane P : *E. coli* HMS 174(DE3) pLysS containing vector alone (pET-28a).
lane Marker : standard markers. Protein standards employed were : myosin (202 kDa), ß-galactosidase (133 kDa), BSA (71 kDa), carbonic anhydrase (41.8 kDa) and soybean trypsin inhibitor (30.6 kDa).

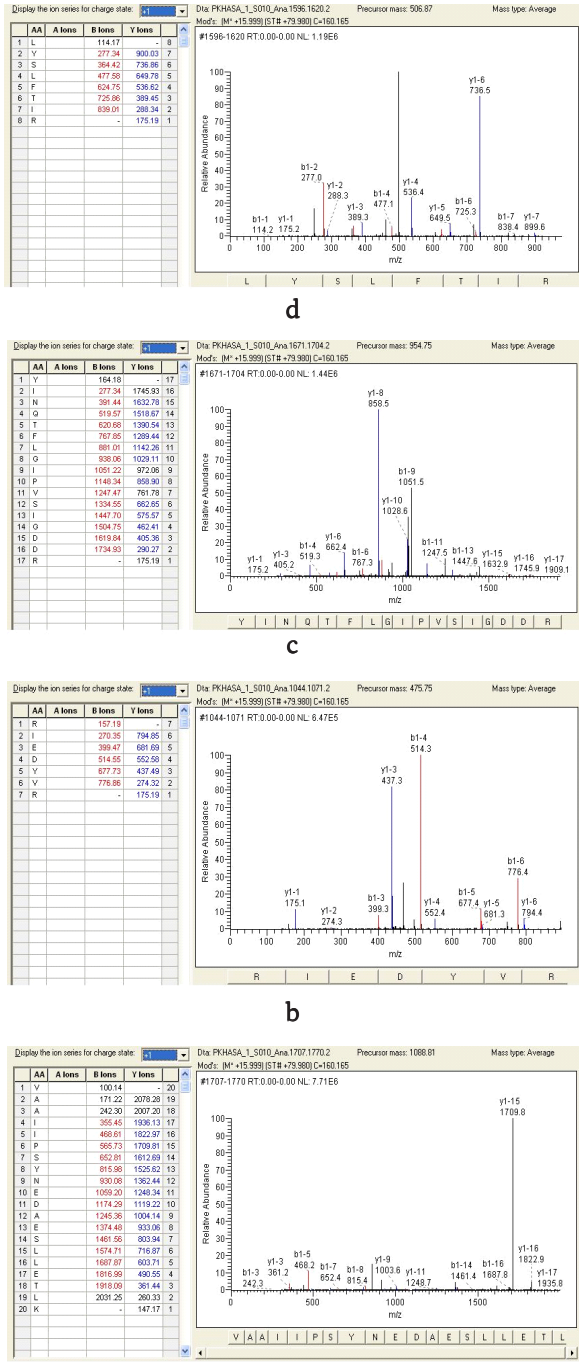

Fig 5. The mass spectra of the fragment ions from LC/MS-MS analysis of the overexpressed streptococcal HAS in *E. coli*. The fragment ions (MS/MS) with the precursor masses of 1,088.81 (Fig. a), 475.75 (Fig. b), 954.75 (Fig. c) and 506.87(Fig. d) were established by sequest software (biowork 31.SR1) to correspond to (VAAIIPSYNEDAESLLETLK, RIEDYVR, YINQTFLGIPVSIGDDR and LYSLFTIR), the amino acid sequence of *Strep. equi* subsp. *zooepidemicus* hyaluronic acid synthase gene in nr fasta database.

Table 1. Hyaluronic acid production from *E. coli* transformants with plasmids harboring the sz-*has*A gene after induction times of 2, 4, 6 and 8 hr.

E. coli strain	plasmid	HA (μg/ml) induction time (hr)				
		0	2	4	6	8
BL21 (codon+)	pET-28 a	9.5	10.4	11.0	10.9	10.1
BL21 (codon+)	pHAS-1	9.3	10.2	16.0	13.2	11.6
HMS 174 (DE 3)	pET-28 a	9.6	10.1	11.2	10.5	10.4
HMS 174 (DE 3)	pHAS-2	9.8	11.6	32.5	24.0	18.2

at the maximum concentration of 16 μg/ml after 4 hr induction time (Table 1). However, the concentration of HA was quite low when compared with the background in the control bacteria (11 μg/ml). In order to increase the HA production level, the plasmid pHAS-2 harboring the sz-*has*A gene with rare codon modifications was constructed. It has been reported that the existence of the rare codons near the 5'-terminus of the gene severely affects the production level of the protein encoded by this gene.[19-22] In the modified sz-*has*A gene, the codons for Arg, Thr and Ile (at amino acid positions 2, 3 and 8) were changed from AGA to CGT, ACA to ACC, and ATA to ATC, respectively, according to Komine *et al*.[23] When the production levels of szHAS from various transformants with pHAS-2 were examined by SDS-PAGE, only *E. coli* HMS 174 (DE3) pLysS transformants produced HA. The production levels from these transformants were higher than that from *E. coli* BL21 (codon+) transformants with pHAS-1, and reached 32.5 μg/ml after 4 hr of induction (Table 1). This study suggests the use of the modified sz-*has*A gene to increase HA production in *E. coli* host.

However, the effects of various culture conditions of HA produced by *E. coli* transformants, such as, temperature, pH and glucose concentration need to be further studied. Competition for substrate and energy resources between catabolic, anabolic, and HA synthesis pathways in the organism should be considered. The metabolic UDP-*N*-acetyl-glucosamine (UDP-GlcNAc), for example, is a common precursor of both HA polymerization and cell wall synthesis.[8] In another point of view, genes cloning and recombinant expression of genes for enzymes which provide some more substrates may increase the production of hyaluronic acid in *E. coli*. Based on previous knowledge, the hyaluronic acid production in *Strep. pyogenes* (group A Streptococci) involves two more enzymes: UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, which are encoded by *has*B and *has*C and provide UDP-GlcNAc and UDP-GlcA, respectively. Therefore, the gene cloning of *has*B and/or *has*C may be required to increase HA production in *E. coli*.

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