Development of a New Type of Recombinant Hyaluronidase Using a Hexahistidine; Possibilities and Challenges in Commercialization

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Hyaluronidases enhance therapeutic drug transport by breaking down the hyaluronan barrier to lymphatic and capillary vessels, facilitating their tissue absorption. Commercially available hyaluronidases are bovine in origin; however, they pose risks such as bovine spongiform encephalopathy. The present study aimed to develop a novel, highly active hyaluronidase and assess its function. Therefore, in order to find the most efficient active hyaluronidase, we produced several shortened hyaluronidases with partial removal of the N- or C-terminal regions. Moreover, we created an enzyme that connected six histidines onto the end of the hyaluronidase C-terminus. This simplified subsequent purification using Ni²⁺ affinity chromatography, making it feasible to industrialize this highly active recombinant hyaluronidase which exhibited catalytic activity equal to that of the commercial enzyme. Therefore, this simple and effective isolation method could increase the availability of recombinant hyaluronidase for research and clinical purposes.

Keywords: Recombinant, hyaluronidase, affinity chromatography, enzyme, hyaluronic acid

Glycosaminoglycan hyaluronic acid (HA), a polymer consisting of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, is a major structural component of connective, epithelial, and neural tissues, and the cumulus-oocyte complex [1–3]. The mechanisms of HA synthesis and degradation are critical to multiple biological processes, including cell migration, wound healing, malignant transformation, tissue turnover, fertilization, and egg development [4–8]. Hyaluronidases, which possess endo-beta-N-acetyl hexosaminidase activity, are responsible for the degradation of HA, and are widely

distributed from bacteria to mammals. Of six human hyaluronidases, three (HYAL1, 2, and 3) are expressed in somatic cells, whereas the other three (HYAL4 and 6, and sperm adhesion molecule 1 (SPAM1)) are expressed in germ cells and human chromosomes 3p21.3 and 7q31.3 [9–12]. The well-known, sperm-specific glycosylphosphatidylinositol-anchored hyaluronidase, SPAM1, has been long believed to assist sperm penetration through the cumulus mass surrounding the eggs [13, 14].

Hyaluronidase is commercially available as an injectable enzyme solution that speeds the natural breakdown of HA.

Table 1. Primer sequences for hyaluronidase fragments.

	Sense primer (5' to 3')	Anti-sense primer (5' to 3')
Short 1	GCCACCATGGACAGCGTGGGCTTGGCGGTC	TTAATTTTAATAGGTTGTTGAT
Short 2	GCCACCATGAGAATGCTGAGGCGCCACCA	TCACTTCACAGGGCTGTCTATAC
Short 3	GCCACCATGAGAATGCTGAGGCGCCACCA	TCACCAGTGTTTCCTTGTACACA
His tagged	GCCACCATGAGAATGCTGAGGCGCCACCA	ATGATGATGATGATGATGTACCTTCACAGGGCTGTCTATAC

It can be administered subcutaneously with other medicines to improve their uptake by the body. Hyaluronidase injection is not only used to increase the absorption of fluids or medicines that are injected subcutaneously, but also to improve absorption of radioactive substances during a procedure called subcutaneous urography. Our previous study demonstrated that bovine SPAM1 is the most active hyaluronidase in mammals [15–17]. SPAM1 exhibits dual functions: hyaluronan-hydrolyzing and ZP-binding activities. Because SPAM1 is enzymatically active at both acidic and neutral pH, the neutral enzyme is postulated to function in hyaluronan degradation of the cumulus cell layer during sperm penetration [10].

Currently, two main types of hyaluronidase are commercially available: one extracted directly from bovine testis and the other, a human recombinant hyaluronidase. Bovine hyaluronidase has merit for its activity, but may convey potential risk of disease via contamination with infectious agents such as bovine spongiform encephalopathy, because of its native animal origin. Alternatively, the recombinant human hyaluronidase produced by expression of the human SPAM1 gene in COS cells requires purification steps and generally shows low activity. Therefore, in this study, we aimed to develop a recombinant hyaluronidase with high activity, low risk of contamination, and at an easy-to-achieve, high level.

Materials and Methods

DNA Cloning

Six oligonucleotide primers were designed to amplify DNA fragments encoding various bovine SPAM1 regions (Table 1). PCR was performed with a PCR thermal cycler (Takara Bio Inc., Japan) for 35 cycles at 94°C for 60 sec, 60°C for 60 sec, and 72°C for 120 sec. The products were cloned, sequenced, and subjected to 1.0% agarose gel electrophoresis. The desired PCR products were

then purified using a gel extraction kit in accordance with the manufacturer's guidelines. Purified DNA fragments were cloned in DH5 α cells using the pMD20 vector kit (Takara Bio., Japan). Following plasmid DNA isolation, inserts were sequenced using the vector-specific sequencing primer on an ABI 3100 DNA sequencer (Applied Biosystems, USA). The DNA fragments were then introduced into a pCXN2 vector. HEK 293 cells were transfected with the plasmid constructs via the Lipofectamine method (Invitrogen, USA) and cultured in Dulbecco's modified Eagle medium/heat-inactivated fetal bovine serum in the presence of 0.5 mg/ml G418 at 37°C in 5% CO₂/95% air. For the HA degradation assay, the G418-resistant clones were dissolved in RIPA buffer after obtaining the supernatant.

Production of Chimeric Hyaluronidase

To construct a new type of recombinant hyaluronidase using human SPAM1 (*hSPAM1*) and bovine SPAM1 (*bSPAM1*) gene information, the plasmid pCXN2-bSPAM1 and -hSPAM1 gene fragments were used as a template (Fig. 3A). After performing the 1st PCR using the above method, the two fragments were ligated with T4 DNA ligase in a total volume of 10 μ l (Elpis Biotech, Korea). The 2nd PCR was subsequently performed with a mixture containing 0.1 μ l of the resulting ligation product, 0.5 μ M of each primer (Table 2), and Pfu polymerase in a total volume of 50 μ l. After a denaturation step at 94°C for 3 min, 35 cycles were carried out at 94°C for 1 min, 60°C for 30 sec, and 72°C for 90 sec. The target DNA fragment was obtained by gel purification of each PCR product inserted into the pCXN2 and sequenced.

His-Tagged Recombinant Hyaluronidase

To express His-tagged bSPAM1 hyaluronidase, DNA fragments encoding bSPAM1 were amplified via PCR, ligated into the pCXN2 vector connected poly-histidine and then transfected into HEK293 cells using Lipofectamine 2000. Cells were then cultured for two days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HEK293 cells expressing the His-tagged proteins were lysed in phosphate buffered saline (PBS) containing 1% Triton X-100 and 1% protein inhibitor cocktail. After

Table 2. Primer sequences for chimeric hyaluronidase.

	Sense primer (5' to 3')	Anti-sense primer (5' to 3')
Human	gccaccatgggagtgctaaaattcaagca	tattacaattccagaagcaccag
Bovine	tggggcagtgctcaatctaagctta	ctagaggtgatgtgatgtgcttcaca

centrifugation (16,000 ×g) for 10 min, the recombinant protein was affinity-purified on a His-binding agarose resin column (Elpis Biotech, Korea), as previously described [8]. The sample was then subjected to SDS-PAGE to analyze the activity of recombinant hyaluronidase through interactions between poly-histidine and Ni²⁺.

Zymography

Proteins exhibiting hyaluronidase activity were visualized via SDS-PAGE in the presence of 0.01% human umbilical cord hyaluronic acid (HA) (Sigma-Aldrich, USA) under non-boiled and non-reducing conditions. After electrophoresis, the gels were washed with 50 mM sodium acetate buffer, pH 7, containing 0.15 M NaCl and 3% Triton X-100 at room temperature for 2 h to remove SDS. The gels were then incubated in the same buffer without Triton X-100 at 37°C overnight. The HA-hydrolyzing proteins were detected as transparent bands against a blue background by staining the gels with 0.5% Alcian Blue 8 GX and Coomassie brilliant blue R-250 [10].

HA Dispersal Assay

Fifty microliters of 1% HA was digested with recombinant hyaluronidase for 12 h in an incubator set at 37°C. Additionally, to ascertain whether the tissues and recombinant hyaluronidase activity patterns vary according to pH, the HA digestion assay was performed over a range of pH values (2–10). The digested hyaluronan samples were then separated via electrophoresis on 0.8% agarose gels. Finally, the gels were stained with 0.5% Alcian Blue 8GX to visualize the HA bands.

Dispersal of Cumulus Cells In Vitro

Superovulation was induced in female ICR mice (8 weeks old, Hyo-Chany Sci., Korea) with an intraperitoneal (IP) injection of

pregnant mare's serum gonadotropin (5 units, Teikokuzoki, Japan) and human chorionic gonadotropin (5 units, Teikokuzoki) 48 h later. The cumulus mass containing eggs was collected from the oviductal ampulla of the superovulated mice 14 h after the injection of human chorionic gonadotropin, placed in a 50-μl drop of TYH medium covered with mineral oil, treated with recombinant hyaluronidase, and then observed under an Olympus (Tokyo, Japan) IX71 microscope equipped with a DP-12 camera, as described [10].

Results and Discussion

To investigate the high activity level of hyaluronidase, we first constructed three fragments with encoding the N- and C-terminal regions (Short 1, 2, and 3), and inserted them into the pCXN2 mammalian expression vector, based on GenBank accession number NM-214011.1 (Fig. 1A). We focused on ways of producing and commercializing the newly reconstituted bovine hyaluronidase, by removing the C-terminal region possessing a very low homology with the human counterpart. A comparison of the homology between bovine SPAM1 and human SPAM1 hyaluronidase amino acids shows 63.55% overall identity, which reaches 66% on removing 80 amino acids from the C-terminal region of bovine hyaluronidase [15]. We have successfully produced three types of fragments. Interestingly, the C-terminus-deleted fragment (Short 2; recombinant (r)bSPAM1^{C80-del}) was active, whereas the N-terminus-deleted fragment (Short 1; rbSPAM1^{N135-del}), and rbSPAM1^{C158-del} (Short 3), were inactive (Fig. 1B).

To examine the potential utility of rbSPAM1^{C80-del}

Fig. 1. Characterization of bovine sperm adhesion molecule 1 (SPAM1) hyaluronidase activity.

(A) Schematic diagrams of several bovine SPAM1 fragments. (B) Four types of fragments were transfected into HEK293 cells. After culturing, the four transfected proteins were incubated with 1% high polymer hyaluronic acid (HA) in phosphate-buffered saline. Samples were separated by 0.8% agarose gel electrophoresis. M; methionine, F; phenylalanine, W; tryptophan, L; leucine, H; histidine. The asterisk and arrow indicate degraded and undigested high polymer HA, respectively.

Fig. 2. Characterization of the His-tagged C-terminal-deleted recombinant bovine SPAM1 fragment (rbSPAM1^{C80-del}-6xH).

(A) Structural representation of rbSPAM1^{C80-del} showing the poly histidine-tag fused to the C-terminus. (B) Expression of rbSPAM1^{C80-del}-6xH. rbSPAM1^{C80-del}-6xH was detected with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% HA by staining with Alcian blue 8 GX. (C) The rbSPAM1^{C80-del}-6xH hyaluronidase was purified using nickel-nitrilotriacetic acid chromatography. The purified protein was analyzed by 10% SDS-PAGE under non-reducing conditions by staining with Coomassie blue. The purified protein is indicated by the arrowhead. (D) Purified hyaluronidase separated by 1.0% agarose gel electrophoresis followed by staining with Alcian Blue 8 GX. The white arrow head indicates digested HA. Control indicates 10 ug of the soluble fraction from transfected HEK 293 cells. (E) Evaluation of commercial possibilities of rbSPAM1^{C80-del}-6xH hyaluronidase. The ability to disperse HA was assessed after incubating rbSPAM1^{C80-del}-6xH with a high polymer of HA. 1; un-cut high polymer HA, 2; sample for rbSPAM1^{C80-del}-6xH, 3; commercial hyaluronidase.

hyaluronidase, and whether it could be produced on industrial scale, we constructed a vector with a 6× histidine-

tag at the C-terminus of rbSPAM1^{C80-del} (rbSPAM1^{C80-del}-6xH) (Fig. 2A). Fig. 2B shows hyaluronidase activity of the

Fig. 3. Construction of chimeric hyaluronidase from human and bovine hyaluronidase.

(A) Schematic representation of bovine and human sperm adhesion molecule 1 (SPAM1). These two proteins contain four putative domains: N-terminal signal peptide, hyaluronidase catalytic, ZP-binding, and C-terminal recognition for attachment to glycosylphosphatidylinositol. Hyaluronidase chimeric cDNAs were generated by converting 339 amino acids of the N-terminus in human SPAM1 to bovine SPAM1. (B) Hyaluronidase purified by nickel-nitrilotriacetic acid chromatography was incubated with 1% high polymer HA in phosphate-buffered saline. Samples were separated by 0.8% agarose gel electrophoresis and stained with Alcian blue. The asterisk and arrow indicate HA degraded by the Short 2 (rbSPAM1^{C80-del}) and chimeric hyaluronidase, respectively.

supernatant obtained after transfection by the aforementioned method, followed by successful purification using a Ni^{2+} column (Figs. 2C and 2D). In addition, as shown in Fig. 2E, rbSPAM1^{C80-del} hyaluronidase displayed HA dispersal ability similar to that of commercial hyaluronidase, indicating its commercial value (Fig. 2E).

Because bovine hyaluronidase has high activity but may have potential side effects, we created a chimeric hyaluronidase by combining the human and bovine proteins, utilizing human hyaluronidase in place of the N-terminus of active hyaluronidase (Fig. 3A). Comparing the efficiency of chimeric hyaluronidase to that of rbSPAM1^{C80-del}-6xH hyaluronidase (Fig. 3B), we found that its activity was lower than that of rbSPAM1^{C80-del}-6xH hyaluronidase but higher than that of human hyaluronidase. We then assessed whether highly purified chimeric hyaluronidase could dissolve commercial HA-based injectable fillers used in cosmetic surgery for soft tissue augmentation, such as anti-wrinkle injections and breast enhancement. Fig. 3B clearly shows that rbSPAM1^{C80-del}-6xH shows excellent resolution of high polymer HA, whereas chimeric hyaluronidase shows low resolution. It is uncertain why the chimera showed a lower enzyme activity than the wild type; however, the structural aspect of the reconstituted hyaluronidase protein might affect the chimera activity. However, further studies need to be performed to elucidate why the chimera showed a lower enzyme activity. The rbSPAM1^{C80-del}-6xH hyaluronidase exhibited an activity level equal to that of the commercial bovine native hyaluronidase in the high polymer HA dispersal assay (Fig. 2E). In addition, we confirmed that rbSPAM1^{C80-del}-6xH can disperse COC (Fig. 4A). We next checked whether the rbSPAM1^{C80-del}-6xH produced in this study was stable at various pH conditions (pH2~pH10). The results showed no activity at pH 2; however, the enzyme was active at other pH values (Fig. 4B).

Side effects of dermal HA filler injections may involve edema or tissue necrosis due to blockage of lymphatic subcutaneous small capillaries. Rapid dissolution of HA fillers by local hyaluronidase injection could mitigate such responses. To confirm the stability of rbSPAM1^{C80-del}-6xH, its ability to hydrolyze HA was verified under various temperature conditions. Polymeric HA decomposition was observed in samples kept at room temperature for seven days (Fig. 4C), as well as in samples that were boiled at 100°C (Fig. 4D). To further explore the characterization of hyaluronidase, hyaluronidase was analyzed by a hyaluronidase inhibition assay by adding 2-mercaptoethanol

Fig. 4. Temperature stability and pH activity testing of recombinant his-tagged hyaluronidase.

(A) Cumulus oocyte complex (COC). COCs were incubated for 60 min with rbSPAM1^{C80-del}-6xH hyaluronidase. Asterisks indicate undispersed COC, whereas the arrowheads indicate oocyte after COC dispersal. (B) An HA digestion assay showed activity from pH 2 to pH 10. (C) rbSPAM1^{C80-del}-6xH hyaluronidase stability evaluation at 37°C at various time intervals and (D) HA degradation reaction with rbSPAM1^{C80-del}-6xH hyaluronidase under 1 mM of 2-mercaptoethanol (2ME) or by boiling. The arrowhead indicates less decomposed HA. An asterisk indicates cleaved HA fragments.

(2ME). When compared with positive control, there was no hyaluronidase activity in the sample with added 2ME, despite the slight hyaluronidase activity in the boiled samples (Fig. 4D).

Thus, our data obtained from the chimeric hyaluronidase construction suggest that the disulfide bonds involved in formation of the protein structure are important for the activity of the recombinant hyaluronidase enzyme. This study was performed to determine whether the newly developed recombinant hyaluronidase showed stable activity at room temperature; if so, the enzyme would have high commercial value. In conclusion, our results suggest that rbSPAM1^{C80-del}-6xH has the potential to rapidly dissolve a high polymer HA-based substance, indicating its effective application in cosmetic surgery, in vitro fertilization, and drug delivery.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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