Industrialization Possibilities of Purified Pig Sperm Hyaluronidase

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Abstract: The goals of the present study were to develop a simple method for obtain highly purified pig sperm hyaluronidase (pHyase) and to assess its activity, function, and safety. In mammals, sperm-specific glycophosphatidylinositol (GPI)-anchored Hyase assists sperm penetration through the cumulus mass surrounding the egg and aids in the dispersal of the cumulus–oocyte complex. Recently, Purified bovine sperm hyaluronidase (bHyase) has been shown to enhance therapeutic drug transport by breaking down the hyaluronan barrier to the lymphatic and capillary vessels, thereby facilitating tissue absorption. Commercially available Hyase is typically isolated from bovine or ovine; which have several disadvantages, including the risk of bovine spongiform encephalopathy, low homology with human Hyase, and the requirement for relatively complex isolation procedures. This study successfully isolated highly purified pHyase in only two steps, using ammonium sulfate precipitation and fast protein liquid chromatography. The isolated Hyase had activity equal to that of commercial bHyase, facilitated in vitro fertilization, and effectively dissolved high molecule hyaluronic acid. This simple, effective isolation method could improve the availability of pHyase for research and clinical applications.

Keywords: epididymal sperm; hyaluronidase; fertilization; cumulus oocyte complex, clinical applications, purification

INTRODUCTION

Hyaluronic acid (HA) is a glycosaminoglycan polymer consisting of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid and is a major structural component of the extracellular matrix and cumulus-oocyte complex (COC) [1–4]. Regulated HA synthesis and degradation are critical in multiple biological processes, including cell migration, wound healing, malignant transformation, tissue turnover, fertilization, and egg development [5–7]. Hyaluronidases, enzymes responsible for HA degradation, are widely distributed in mammals [8,9]. These enzymes exhibit endo-beta-N-acetyl hexosaminidase activity and produce tetrasaccharides and hexasaccharides as the major end products of HA degradation. The three pig genes encoding the hyaluronidases (Hyase), *HYAL1*, *HYAL2*, and *HYAL3*, are clustered on chromosome 13p21.3, whereas the genes encoding *HYAL4*, *HYAL6*, and *HYAL7* are clustered on chromosome 7p31.3 [10–12]. Among these Hyase, the sperm-specific Hyase, HYAL7, facilitates the penetration of sperm through the COC containing a metaphase II-arrested oocyte surrounded by the zona pellucida (ZP). HA is embedded in the extracellular matrix, which is abundant in COC and its degradation is necessary for fertilization [13]. Although its function and safety remains unresolved, HYAL7 Hyase is frequently used in cosmetic procedures and for *in vitro* fertilization (IVF). Commercial Hyase, isolated from bovine or ovine testis extracts, has long been used to increase the absorption of drugs into tissue and to reduce tissue damage in case of drug extravasation. With the increasing popularity of HA filler, Hyase has been essential drug for the correction of complications and unsatisfactory results after filler injection. However, both currently available commercially bovine sperm hyaluronidase (bHyase) has approximately 55% amino acid homogeneity with human sperm hyaluronidase (hHyase), and thus, may have potential side effects.

Throughout human history, animal by-products have been partially commercialized, but mostly abandoned. Fetal bovine serum has been used in cell culture research since a century ago, and has since been used in the dermatology field along with HA extracted from chicken comb [14,15]. Collagen, the most abundant protein in mammals, is the main structural protein of the extracellular matrix found in various connective tissues in the body. Notably, the collagen used in the medical and cosmetic field is

derived from the skin of cows and pigs [16]. Pigs are an excellent model for understanding human diseases because their anatomy and physiology closely resembles those of humans, which is not the case with other experimental animal models. Hence, pigs are extensively used as general surgical models and in transplantation and xenograft research. Pigs are also widely available for human protein supplementation in many countries, and are raised worldwide for pork consumption. However, during slaughter, most organs other than the meat (flesh), are discarded. In this study, we attempted to extract hyaluronidase from the epididymidis, a discarded pig by-product, and examined its industrial value. Thus, we purified and characterized a high-quality pig sperm hyaluronidase (pHyase) using a simple two-step process, and demonstrated its high activity and safety for research and clinical use.

MATERIALS AND METHODS

Tissue sample collection and preparation of protein extracts

Fresh porcine and bovine epididymides were purchased from a local slaughterhouse. The samples were immediately flushed with ice-cold buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4). Epididymal sperm were extracted by mincing and squeezing the porcine and bovine epididymis in a buffer containing 20 mM Tris-HCl (pH 7.4) 1% Triton X-100, 150 mM NaCl, and a 1% protease inhibitor cocktail (Millipore, Burlington, MA, USA) and kept on ice for 2 h. The suspensions were then centrifuged at 10,000 g for 10 min at 4 °C. The concentration of sperm extracts were determined using the Bradford method. All experiments were approved by the Institutional Animal Care and Use Committee of Daegu-Gyeongbuk Medical Innovation Foundation (Daegu, Korea; Approval No. DGMIF-21021602-00).

Purification of porcine sperm hyaluronidase

Thirty gram of ammonium sulfate were added to 100 mL pig epididymal sperm extracts to react at 4 °C for 2 h. The mixture was centrifuged at 10,000 g for 5 min, and the pellet was separated. Thereafter, 10 g of ammonium sulfate was added to the supernatant liquid to make it 40 g, reacted at 4 °C for 2 h,

centrifuged, and the pellet was separated [17]. Subsequently, the pellet was separated while adding 10 g, 5 g, 5 g, and 10 g, 10 mL of 150 mM sodium chloride solution was added to each pellet, dissolved, and dialysis was performed three times to obtain ammonium sulfate fractions. The 55% ammonium sulfate fraction was applied to a Hi-Trap heparin HP column (cat. #17-0407-03, Amersham Pharmacia Biosciences, Uppsala, Sweden) that had been equilibrated with 20 mM Tris/HCl (pH 7.5). Proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 0.5 mL/min [18]. Aliquots of each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of hyaluronan, as described below.

SDS-PAGE and Zymography

Proteins with Hyase activity were visualized using SDS-PAGE in the presence of 0.02% high molecular weight hyaluronan 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. Next, the gels were incubated overnight in the same buffer without Triton X-100 at 37 °C. Hyaluronan-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 [19,20].

In vitro maturation of oocytes

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 °C in 0.9% saline supplemented with 75 μg/mL potassium penicillin G and 50 mg/mL streptomycin sulfate. COCs were aspirated from follicles with a 3–6 mm diameter into a disposable 10 mL syringe by using an 18-gauge needle. After three washes with HEPES-TL medium [23], approximately 50 oocytes were matured in 500 μL of in vitro maturation medium in a four-well dish (Nunc, Roskilde, Denmark) at 38.5 °C and 5% CO_2 in the air. The NCSU-23 medium supplemented with 10% follicular fluid, 0.57

mM cysteine, 10 ng/mL β-mercaptoethanol, 10 ng/mL epidermal growth factor, 10 IU/mL pregnant mare serum gonadotropin, and 10 IU/mL human chorionic gonadotropin was used for oocyte maturation. In addition, 10 ng/mL of estradiol (E2) was added to the maturation medium of the experimental samples during the initial maturation step. After 22 h of culturing, the oocytes were washed thrice and cultured for an additional 22 h in the maturation medium [21] without supplementation with either hormone [22].

Dispersal activity of porcine COCs.

The maturated porcine COCs were placed in a 50 µl drop of TYH medium modified Krebs-Ringer bicarbonate solution (supplemented with glucose, Na-pyruvate, antibiotics and bovine serum albumin) and covered with mineral oil, treated with purified porcine sperm hyaluronidase or commercial bull hyaluronidase for 30 min and then observed under an Olympus IX71 microscope (Tokyo, Japan) equipped with a DP-12 camera.

In vitro fertilization assay

For the IVF assay, a modified Tris-buffered medium (mTBM; 113.1 mM NaCl, 3mM KCl, 7.5mM $CaCl_2 \cdot 2H_2O$, 20mM Tris) was prepared. Freshly ejaculated semen was washed thrice through centrifugation with Dulbecco's phosphate buffered saline (Gibco-BRL, Grand Island, NY, USA) supplemented with 1 mg/mL bovine serum albumin (BSA), 100 µg/mL penicillin G, and 75 µg/mL streptomycin sulfate. After washing, the spermatozoa were suspended in mTBM (pH 7.8). The oocytes were washed thrice in mTBM with 2.5 mM caffeine/sodium benzoate and 4 mg/mL BSA (fatty acid-free), and then placed in 50 µL mTBM under paraffin oil. Diluted spermatozoa (2 µL) were added to 50 µL mTBM containing 15−20 oocytes to give a final concentration of 1.5×10^5 sperm/mL. The oocytes were incubated with the spermatozoa for 6 h at 38.5 °C in an atmosphere of 5% CO_2 in the air. Eggs were denuded by gentle pipetting in mTBM containing 4% formaldehyde at 4 °C. The cells were

then washed with PVA- phosphate buffered saline (PBS) and mounted on slides. The samples were then fixed with acetic acid for 10 min [22]. The number of sperm bound per egg was counted using a microscope at 200 magnification (Leica GmbH, Wetzlar, Germany).

Statistical analyses

All data are representative of at least three independent experiments unless otherwise stated. The results are expressed as the mean ± standard error of mean. The Student's *t-test* and one-way analysis of variance followed by Duncan test were used for statistical analyses. Effects were considered statistically significant if $P< 0.05$.

RESULT AND DISCUSSION

Although whether sperm-specific Hyase is essential for mammalian fertilization remains unresolved [20], experiments demonstrated that Hyase increases tissue permeability. Commercially available hyaluronidase is derived from rams and bulls, representative livestock that are an invaluable protein source worldwide, and recombinant human Hyase has been clinically used in conjunction with other drugs to speed their dispersion and delivery.[23–25]

In our study, we examined whether there would be commercial value in extracting hyaluronidase from pig epididymal sperm, a commercial by-product of pig slaughter. The disadvantage of commercially available bHyase is that it has a potential risk of transmitting bovine spongiform encephalopathy disease, whereas sheep are raised less frequently than cattle or pigs and thus, are not a viable source. However, pigs are the most consumed livestock in south Korea, and pork consumers generally prefer the meat (flesh), leading to all the other organs and parts being discarded as by-products. Considering the potential disadvantages of bovine- and ovine-derived Hyase preparations and the abundant availability of pork by-products, we developed a purification process to extract Hyase from

porcine epididymal sperm. Our first experiment confirmed that pig sperm Hyase (pHyase) demonstrated expected enzymatic activity and had commercial value. The activities of Hyase preparations from epididymal sperm extracts of mouse, pig, and bull were compared to determine their utility (Figure 1A).

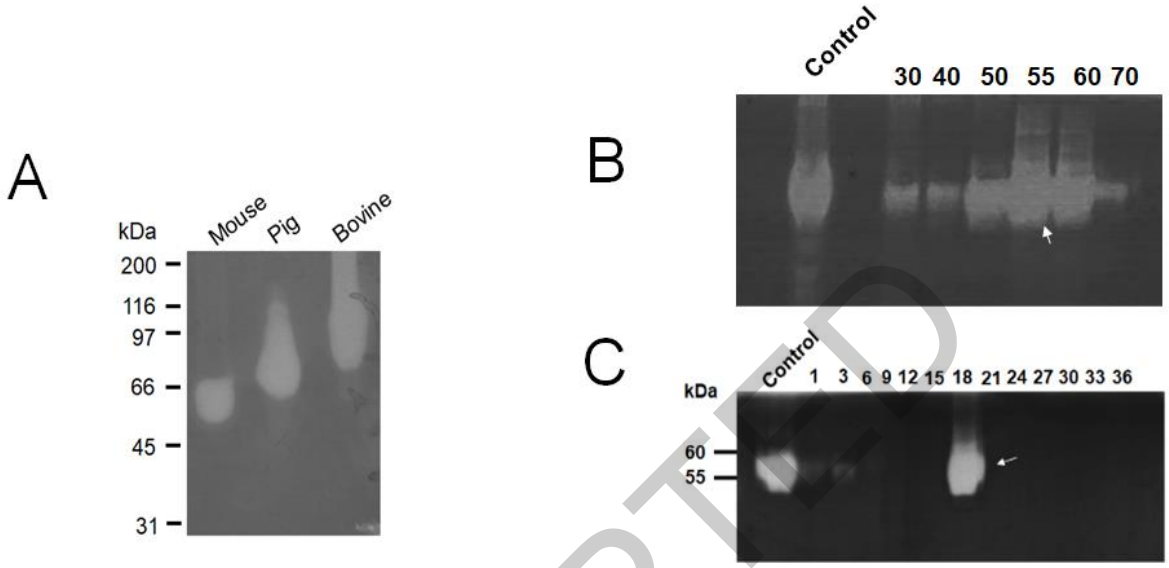

Fig. 1. Presence of sperm hyaluronidase in various species and two-step purification of porcine epididymal Hyase. (A) Epidydimal sperm extracts were separated by electrophoresis in an 8.5% sodium dodecyl sulfate–polyacrylamide gel under non-reducing conditions and analyzed using hyaluronan zymography. (B) The 55% ammonium sulfate fraction from porcine epididymal extracts demonstrated the strongest hyaluronidase activity (arrow). Numbers mean for the amount of ammonium sulfate contained in 100 ml. (C) The positive band from the 55% ammonium sulfate fraction was applied to an affinity column of heparin–sepharose (5 mL). The enzyme was detected at lane 18 in the final purified product (arrow). Commercial hyaluronidase was used as control. Numbers refer to the fraction purified from Hi-Trap heparin HP column.

The findings confirmed that Hyase shows strong activity at approximately 55 kDa and is active in several species.Interestingly, Hyase activity varied among different animals during the zymography assay even though the same concemtration of sperm extracts was used. Porcine and bovine sperm extracts had Hyase activity of similar strength, whereas mouse extracts showed relatively low activity. To examine the potential utility of pHyase and determine whether it can be isolated on an industrial

scale, pHyase was isolated from pig epididymal sperm extract using a two-step purification method (Fig. 1B). Treatment with 55–60% ammonium sulfate yielded a precipitate that retained Hyase activity. The Hyase was further purified through dialysis of this precipitate against 1× PBS followed by fast protein liquid chromatography with a Hi-Trap heparin column. The fraction with the highest activity (No. 18) was eluted with approximately 20 mM NaCl (Fig. 1C). In this study, we demonstrated high hyaluronidase activity using only two steps. In order to confirm the ability of purified pHyase to disperse high-polymer HA, the enzyme was added to 1% high molecular weight hyaluronic acid, incubated at 37 °C for 12 h, and then subjected to agarose. pHyase successfully decomposed high-polymer HA (Fig. 2A). Therefore, this study highlights the possibility of using discarded pork by-products to produce useful substances, which would improve economic feasibility of their extraction and reduce their environmental impact.

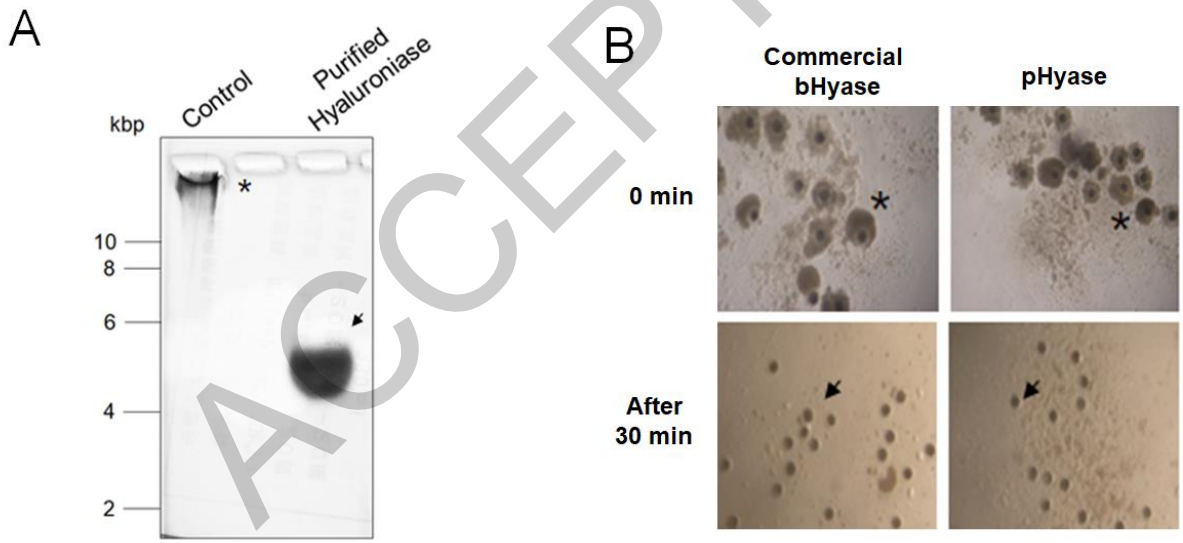

Fig. 2. Hyaluronic acid degradation assay with purified pHyase. (A) After purifying, the sample was incubated with 5% high polymer hyaluronic acid in phosphate-buffered saline. Samples were separated using electrophoresis in 0.8% agarose gel. Asterisk indicates high molecular weight hyaluronan, and the arrow head indicates degraded hyaluronan. (B) Dispersal activity of porcine cumulus-oocyte complexes (COCs). The COCs were incubated for 30 min with purified porcine hyaluronidase (pHyase) or commercial bovine hyaluronidase (bHyase).

198 The second objective of this study was to examine whether highly purified pHyase can be used for
199 research and clinical purposes. We compared the efficiency of pHyase to that of commercial bovine
200 sperm hyaluronidase in COC dispersal (Fig. 2B). When the purified pHyase obtained in this study was
201 added to pig COCs, the COCs were clearly dispersed. Recently, IVF technology and the development
202 of disease models has advanced due to the increase in patients with infertility. Typically, IVF
203 experiments begin with the COC dispersion. The bHyase is generally used for this COC dispersion step,
204 likely due to its obtainability and high activity. Yet, bHyase is relatively complex to isolate, has low
205 homology with human Hyase, and carries the risk of bovine spongiform encephalopathy. However, our
206 findings indicate that pHyase is easily extracted and purified, and is also highly active, and can therefore
207 be an alternative to bHyase for IVF. Figure 2B confirms that no significant difference occurred in COC
208 dispersion ability from purified pHyase and commercially available bHyase (Fig. 2B).

209 Subsequently, the difference between both the Hyase were compared using IVF experiments conducted
210 on pig oocytes. There were no significant differences between the two groups, but IVF was found to be
211 more efficient when using pHyase compared to commercial bHyase (Fig. 3A).

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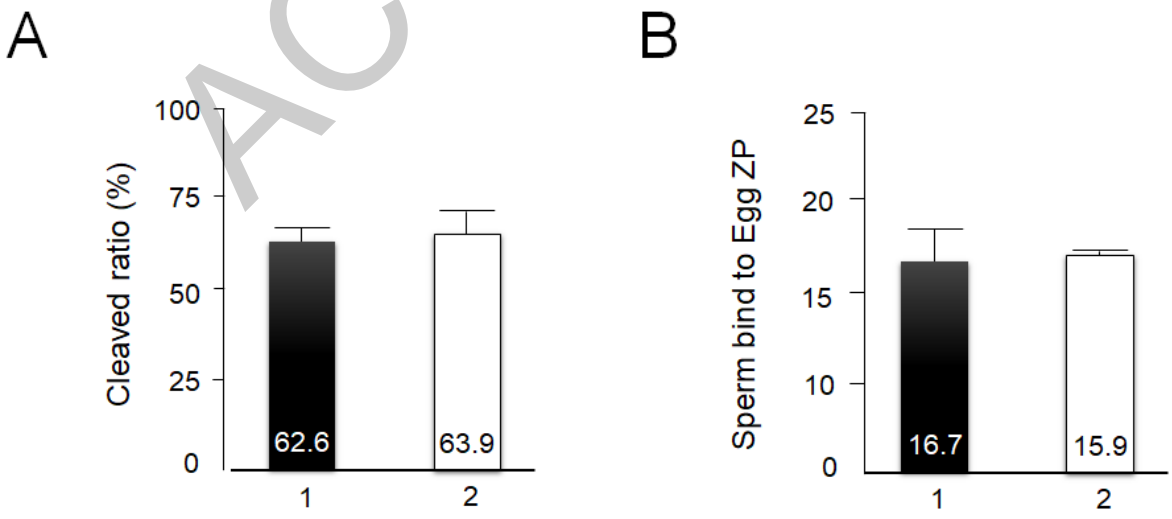

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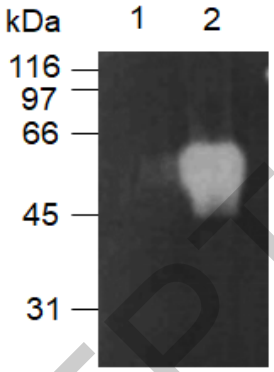
214 Fig. 3. Effects of purified pig sperm hyaluronidase during in vitro fertilization. Cleavage rates (A) and
215 the number of sperm cells bound to zona pellucida (ZP) of pig cumulus-free eggs (B) were not

statistically different after treatments with commercial bHyase (1) and purified pHyase (2). Data are presented as the mean ± standard error of the mean of three independent experiments.

The number of sperm bound to egg in the IVF with pHyase is lower than the case of the commercial use of bHyase, although there is no significant statistical difference (Fig. 3B). For evaluation of IVF efficiency in pig, polyspermy is the primary factor for diminishing the successful fertilization. The relatively low success rate in the pig IVF would be due to that polyspermy occurs more frequently in the pig than in the other animals. Pigs are getting more appreciation as experimental animals used for biomedical research, in that they have many similar characteristics shared with humans, especially in the physio-anatomical aspects. Thus, the improvement of pig IVF efficiency with the techniques specified in this study would provide a new helpful opportunity for the fields of pig research, such as development of disease models, in addition to pig production industry. Therefore, if pHyase can be commercialized, polyspermy occurring as a result of IVF may be less frequent. Further research is needed to determine whether pHyase obtained by our suggested method can be equally efficient in IVF of mice and humans. Collectively, the results of this study prove that pHyase obtained by our proposed method can be considered for commercialization.

Previously, we succeeded in cloning the bHyase gene *bHYAL7*, which demonstrated high activity upon expression in HEK293 cells, unlike mouse sperm Hyase [26]. It should be noted that pigs and humans have a single sperm Hyase gene, whereas rodents and bovines have two, *HYAL5* and *HYAL7*, and the homology rates between corresponding rodent and humans Hyase genes are ~55% [10,11]. The pHyase activity in our study was as high as that of the commercial bHyase. To further investigate pHYAL7, we cloned the cDNA encoding the entire *pHYAL7* sequence based on the GenBank accession number NM-214011.1. The DNA sequence indicated that pHYAL7 was initially synthesized as a single-chain protein consisting of 525 amino acids, with a calculated molecular mass of 59,970 Da. It possessed 25 additional residues at the C-terminus compared to the sequence of human HYAL7, indicating ~67% sequence identity, whereas the identity between human and bovine HYAL7 is 63%. When the cloned

242 *pHYAL7*–pCXN2 vector was expressed in Chinese hamster ovary (CHO) cells, it showed strong
243 zymography activity (Fig. 4). Previously, when mouse HYAL5 and HYAL7 were expressed in CHO
244 cells, no activity was observed (data not shown). Currently, only human HYAL7 (hHyase) has been
245 commercialized as a recombinant Hyase. The advantage of recombinant pHyase is its increased enzyme
246 activity compared to that of recombinant hHyase, and possibility of stable production under clean
247 laboratory conditions. If recombinant pHyase has no side effects, it can be expected to have a significant
248 commercial value.



249

250 Fig. 4. Activity of pHYAL7 in Chinese hamster ovary (CHO) cells. Proteins in Triton X-100 extracts
251 from pHYAL7-transformed CHO cells were separated by sodium dodecyl sulfate–polyacrylamide gel
252 electrophoresis under non-reducing conditions and subjected to a zymography assay. 1, pCXN2 vector;
253 2, pHYAL7–pCXN2 vector.

254

255 CONCLUSION

256 To the best of our knowledge, this is the first study to report the commercial value of pHyase. For the
257 first time, we have purified high-quality pHyase from porcine epididymal sperm extracts using a
258 relatively simple, two-step method. The activity of the obtained pHyase was similar to that of the
259 commercially available bHyase. Furthermore, the porcine *HYAL7* gene inserted into the pCXN2 vector
260 and expressed in CHO cells generated protein product that demonstrated high enzymatic activity. In
261 addition, the use of pHyase was associated with nominally higher cleavage rate and lower extent of

polyspermy during IVF compared to those observed after the use of commercially available bHyase. Collectively, the results of our study indicate that pHyase obtained by our suggested method may be safely and effectively applied in IVF, cosmetic surgery, and drug delivery.

Author Contributions and Conceptualization, S.P and I.M.; methodology, and E.K; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The author declares no conflict of interest.

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