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A method of obtaining highly active hyaluronidase on an industrial scale

Magdalena Matwiejczyk[a]*, Monika Besman[a]*, Filip Porzucek[b], Dominik Ciura[a]

[a] Research and Development Department – Pharmfactor Sp. z o.o., Wrocław, Poland.
[b] Center for Advanced Technology, Adam Mickiewicz University, Poznan, Poland.

*Corresponding author. Email: m.matwiejczyk@pharmfactor.pl, m.besman@pharmfactor.pl

Abstract: Hyaluronidases refer to a group of enzymes that catalyze the hydrolysis of certain complex carbohydrates such as hyaluronic acid and chondroitin sulfates. It is used for its approved indications in such medical fields as orthopedics, surgery, ophthalmology, oncology, and dermatology, generally to increase the absorption of drugs into tissues. At present, market feedback indicates that there are cases of shortage of medicinal products based on high-quality hyaluronidase. This article presents an economical method of obtaining hyaluronidase on an industrial scale yielding a product with a high specific activity that does not require the use of dangerous substances or complicated and expensive techniques.

Keywords: bovine testicular hyaluronidase, highly active hyaluronidase, ion exchange chromatography, large scale production

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1. Introduction

Hyaluronidase is an enzyme (endo-β-N-acetylhexosaminidase) that degrades glycosaminoglycans: hyaluronic acid (digests the glycosidic bonds between N-acetyl- β-D-glucosamine and D-glucuronic acid), chondroitin, chondroitin IV and VI sulfites, and to a varying degree (depending on origin) dermatan sulfate [Lyon & Phelps, 1981; Kaya et al., 2015]. The final reaction products are tetra- and hexasaccharides with an N-acetylhexosamine residue at the reducing end and a glucuronic acid residue (GlcUA) at the non-reducing end [Csóka et al., 1997; Kaya et al., 2015].

Hyaluronidases can be classified according to various criteria [Csóka et al., 1997; El-Safory et al., 2010; Jung, 2020]. In terms of optimum pH, two types can be distinguished: active in an acidic and neutral environment, while in terms of amino acid sequence homology - eukaryotic and prokaryotic. Three types are distinguished based on the mechanism of action: from testicles, leeches, and bacteria. The bacterial mechanism of degradation differs significantly from the others [El-Safory et al., 2010].

The testicular type is found in mammalian testes (mainly sperm), mammalian lysozymes, submandibular glands, and in the venom of snakes, bees, etc. [Csóka et al., 1997]. Six types have been identified in humans: hyaluronidase 1-4, PH-20, and HYALP1 [Csóka et al., 2001; Stern & Jedrzejas, 2006]. PH-20 is specific to testes and plays an essential role in fertilization. Located on the anterior surface of the sperm head, it allows it to reach the egg by penetrating a mass rich in hyaluronic acid [Lin et al., 1994]. This strategy is also used by viruses and parasites during infection. The activity of hyaluronidase during the "invasion" of the cell is that it destroys the integrity of the tissue, and also digests hyaluronic acid found in the extracellular and intracellular matrix [Csóka et al., 1997].

Hyaluronidase increases cell membrane permeability by reducing viscosity and makes tissues more easily permeable to injected fluids [El-Safory et al., 2010].

Table 1. Specific activity increase in the process

Step	Specific activity [IU/mg]	Hyaluronidase quantity [MIU]	Yield [%]
Extraction	42	354.6	100.00
Precipitation of ballast compounds	71	302.6	85.34
I concentration	205	277.2	78.16
I salting-out	224	253.0	71.34
Ultrafiltration	232	238.2	67.16
I chromatography	1776	208.8	58.88
II concentration	1868	201.7	56.89
II salting-out	2237	184.0	52.04
Hyaluronidase precipitate dissolved	4322	183.7	51.80
II chromatography	15052	75.3	21.22
III concentration	15086	71.3	20.10
Microfiltration	15096	71.3	20.09

Abbreviations: IU – international unit

Its ability to facilitate the penetration and spread of substances has found use in many clinical applications. The US FDA has approved 3 uses of hyaluronidase: 1) subcutaneous fluid infusion 2) as an adjuvant to facilitate the absorption and dispersion of drugs in subcutaneous tissue, or to deal with extravasation 3) as an additive to facilitate the absorption of a contrast agent in urinary tract angiography (subcutaneous urography). In Europe, hyaluronidase is additionally used to increase the absorption of hematomas. Applications beyond those indicated include dissolution of hyaluronic acid fillings, treatment of reactions to granulomatous foreign bodies, and treatment of skin necrosis associated with filling injections [Jung, 2020].

The half-life of hyaluronidase in the subcutaneous tissue is less than 30 minutes and in plasma less than 3 minutes. The reason for such a short activity in the plasma is the presence of numerous inhibitors, as well as the breakdown of hyaluronidase by the kidneys and liver [Jung, 2020]. Hyaluronidase inhibitors are glycosaminoglycans: heparins and some dermatan sulfates [Lyon & Phelps, 1981]. Many drugs affect the activity of hyaluronidase, including anti-inflammatory drugs, plant compounds (flavonoids, antioxidants), antihistamines, vitamin C, heparin [Jung, 2020].

Highly purified hyaluronidase is much less stable than unpurified hyaluronidase. In an aqueous solution of less than 1 mg/ml, inactivation occurs, which can be prevented by adding gelatin, gelatin polypeptides, or albumin. Crucial to stability are the pH range, ionic strength, temperature, and concentration of the solution. Purified hyaluronidase cannot be dialyzed against water because it loses 50% of its activity – dialysis against 0.9% NaCl is preferred [Freeman & Webster, 1952].

There is a rich diversity in contemporary ways of obtaining hyaluronidase. Two well-established methods are the production of recombinant protein in bacterial cells and its purification from extracts of animal tissues [Lyon & Phelps, 1981; Balasubramanian et al., 1975; Harrison, 1988; Srivastava et al., 1979; Yang et al., 1975; Barsukov et al., 2003; Baev, 1964; Igonina et al., 1998]. Hyaluronidase is obtained from various tissues of mammals, but most of it is in the testicles and semen. The isolation of hyaluronidase from bovine testes is carried out by chromatographic techniques, primarily affinity chromatography [Lyon & Phelps, 1981; Balasubramanian et al., 1975; Harrison, 1988; Srivastava et al., 1979; Yang et al., 1975; Rongxiu et al., 2013], ion-exchange chromatography [Harrison, 1988; Yang et al., 1975; Barsukov et al., 2003; Baev, 1964] and chromatography on macroporous sorbents [Igonina et al., 1998]. There are also known methods of preparation with the use of organic solvents [Tint et al., 1950; Freeman & Monroe, 1950].

Contemporarily available methods of obtaining hyaluronidase have limitations affecting their efficiency (also financial) and product safety. Firstly, flammable solvents, such as acetone [Yoshida, 1978], are routinely used. The application of flammable solvents on a large scale is associated with numerous complica-

Table 2. Highly active hyaluronidase specification.

Parameter	Specification	Result
Characteristic	Amorphous powder, hygroscopic, of white or yellowish colour	Meet requirements
Identity	Quantitative study of decrease in hyaluronic acid salt viscosity	Meet requirements
Solubility	Soluble in water but practically insoluble in ethanol and acetone	Meet requirements
Appearance of solution (0.1g/10 ml of water)	The solution is clear	Meet requirements
Activity	Not less than 300 IU/mg	14 341 IU/mg
Loss on drying	max 5.0 %	2.5 %
pH of the 0,30% aqueous solution	4.50–7.50	7.30
Bacterial endotoxins	<0.2 IU/1 Ph.Eur.U. of Hyaluronidase	Meet requirements

All parameters were tested according to European Pharmacopoeia methods.

tions and requires additional protection. Secondly, many ways of obtaining hyaluronidase use complex and unprofitable industrial-scale affinity chromatography [Lyon & Phelps, 1981; Balasubramanian et al., 1975; Harrison, 1988; Srivastava et al., 1979; Yang et al., 1975; Rongxiu et al., 2013]. In this way, some methods, despite their high efficiency, may not be suitable for industrial use in practice due to cost and complexity.

This article describes the method of obtaining hyaluronidase from bovine testicles, which does not use dangerous (e.g. flammable) substances or complicated and expensive techniques (in particular affinity chromatography), and at the same time enables to obtain a stable product with sufficient efficiency, safety, and high specific activity.

2. Materials and methods

250 kg of bovine testicles were extracted with an aqueous solution of acetic acid (755 l) with continuous cooling in the enameled tank with an anchor agitator. Precipitation of ballast compounds was achieved using $CaCl_2$. The extract was filtered on a sieve separator and the filtrate was directed to a disc centrifuge (GEA ASE 20-06-077). The supernatant was placed in a tank with a cooling jacket and filtered using ceramic filters. The extract was concentrated using a hollow fiber filter with a cut-off of 30 kDa (Cytiva UFP-30-C-75). The ballasts were precipitated with ammonium sulfate. The solution was centrifuged (Sorvall LYNX 6000), desalted, and adsorbed onto the chromatographic column – a strong cation exchanger. Hyaluronidase was eluted with a linear gradient of ammonium acetate. In subsequent stages, the enzyme was precipitated with ammonium sulfate and subjected to chromatographic separation on Sephacryl S200. The fractions containing hyaluronidase were concentrated and microfiltered. The drying of the final solution was carried out in a freeze dryer. Hyaluronidase activity and other parameters were determined using the pharmacopeia methods (using certified reagents).

3. Results

During the whole process, enzymatic activity and protein concentration were monitored. Summary of received results is presented in Tab.1. The biggest relative specific activity gain was observed in the „chromatography I" and the biggest absolute gain in „chromatography II" step. Chromatography II is also the least effective purification step. Finally, after lyophilization 43.5 g of hyaluronidase with a specific activity of 14,341 U/mg was obtained from 2500 kg of waste meat raw material. The received product was thoroughly characterized according to pharmacopeia monograph. Obtained hyaluronidase meets all European Pharmacopoeia requirements, which is summarized in Tab. 2.

4. Conclusions

The developed manufacturing method omits affinity chromatography, which is not optimal from the economical point of view. It also does not use organic solvents at any stage, which lowers the requirements for a manufacturing facility in terms of safety and is beneficial for the environment. Use of CaCl2 in the precipitation stage significantly shortened this process and improved final product solubility.

In accordance with the method presented, we obtained a product with a high specific activity of 14,000–15,000 U/mg with a total yield of approx. 20%. Moreover, the obtained product meets all the requirements of a Ph. Eur. hyaluronidase monograph.

The developed method of obtaining hyaluronidase became the basis for applying for an international patent (patent application number: P.435319). As part of the study of patentability, a group of over a thousand documents was found, from which eight were selected as the most important in terms of the method of obtaining used [Pope & Baxendale, 1981; Pak & Oficerow, 1992; Craig & Chesham, 2004; Chunying & Liyun, 2011; Qunli et al., 2013; Rongxiu et al., 2013; Woogu et al., 2014; Woo et al., 2017]. According to the results of the International Search Report and the opinion of the European Patent Office, the method of obtaining hyaluronidase developed by the authors of this publication meets the criterion of novelty. At the same time, the cost-effectiveness of the described process may allow it to be used on a large scale, thus meeting the huge market needs associated with numerous applications of hyaluronidase.

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