Horizon 2020 Work programme
Food Security, Sustainable Agriculture and Forestry, Marine, Maritime and Inland Water Research and the Bioeconomy
Call
H2020-FNR-2020: Food and Natural Resources
Topic name
FNR-16-2020: ENZYMES FOR MORE ENVIRONMENT-FRIENDLY CONSUMER PRODUCTS

FuturEnzyme:
Technologies of the Future for Low-Cost Enzymes for Environment-Friendly Products
Final ID: 101000327

21/09/2021

BIBLIOGRAPHIC AND PATENT SEARCH: PRODUCTION OF HYALURONIC ACID FOR COSMETICS

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Summary

BIBLIOGRAPHIC AND PATENT SEARCH: PRODUCTION OF HYALURONIC ACID FOR COSMETICS

1. Introduction

The **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)** requested a background search regarding enzymatic production methods of hyaluronic acid for cosmetics, with the aim of making those patent and non-patent documents that are part of the state of the art related to this technology available to the Center.

A "STATE OF THE ART" report (Phase I) was sent to the CSIC, which included 169 inventions related to the enzymatic production methods of hyaluronic acid for cosmetics.

CSIC reviewed this STATE OF THE ART report (Phase I) and selected 19 patent documents and 17 scientific literature documents for further study. In this study, the technical conditions used in the processes described in the selected documents will be analysed, including the enzymes that are used and their conditions.

2. Patent documents

Table 1 shows a summary of the organisms and enzymes described in each patent document that are used to produce hyaluronic acid, as well as the most important methods and conditions.

Table 1. Organisms, enzymes, methods and conditions of each patent document.

	PUBLICATION NUMBER	ORGANISM / ENZYME	METHODS / CONDITIONS
1	US2011281817 AA	*Streptococcus* cells. Enzymes (Group I): phosphoglucoisomerase, D-fructose-6-phosphate amidotransferase, phosphoglucosamine mutase, glucosamine-1-phosphate acetyl transferase, N-acetylglucosamine-1-phosphate pyrophosphorylase, glucosamine-6-phosphate acetyl transferase, and phosphoacetylglucosamine mutase Enzymes (Group 2): UDP-N-acetylglucosamine 1-carboxyvinyltransferase and undecaprenyldiphospho-	The activity or amount in the cells of one or more enzymes selected from Group I has been increased. The activity or amount in the cells of one or more enzymes selected from Group 2 has been decreased. Substrates selected from UDP-N-acetylglucosamine, N-acetylglucosamine and glucosamine

		muramoylpentapeptide beta-N-acetylglucosaminyltransferase	
2	US2015175991 AA	*Bacillus sp.*, which has a deposit access number CGMCC NO. 5744. Enzyme: hyaluronidase	1. Preparing the solution of hyaluronic acid or its salts 2. Enzymolysis (35 °C.-45 °C., the pH is 5.5-7) 3. Inactivation 4. Filtration 5. Precipitation 6. Dehydrating and drying Optimal temperature: 42℃ Optimal pH: 6.5
3	US2019224232 AA	*Streptococcus* (*Streptococcus equi* sub specie equi 68222, mutant H-1.) or *Bacillus* or rooster combs. Enzymatic digestion (does not specify which enzymes)	1. Inactivation 2. Extraction 3. Purification 4. Thermal treatment
4	CN112501088 A	*Bacillus CQMU-D (Bacillus sp. CQMU-D).* Enzyme: hyaluronidase	1. Activate the preserved *Bacillus* strain 2. Inoculate the fresh strain obtained in step 1 into sterilized seed culture medium 3. Inoculate the seed solution cultured in the step 2 into a sterilized fermentation medium 4. Centrifuge 5. Re-dissolve the crude protein collected in the step 4

5	US2009312283 AA	It does not specify the enzyme (only indicates an enzyme having HA deacetylase activity).	Chemical or enzymatic treatment to deacetylated one or more N-Acetyl-Glucosamine to Glucosamine to form a branched hyaluronic acid. pH conditions: 6-8
6	JP1272511 A2	Testicular hyaluronidase or bacterial hyaluronidase	Describes a cosmetic additive comprising oligosaccharides obtained by treating hyaluronic acid or a salt thereof with testicular hyaluronidase or bacterial hyaluronidase (See the abstract of the document for details)
7	CN110331178 A	*Arthrobacter globiformis* Enzyme: hyaluronidase	1. Prepare the hyaluronic acid 2. Digestion (pH 5-10 and Tª 20-50°C) 3. Inactivation 4. Filtration 5. Precipitation 6. Dehydration
8	US2021198620 AA	*Enterobacter sp*. Enzyme: hyaluronidase	A method of producing hyaluronidase comprising subjecting the Enterobacter CGJ001 to plate culture, seed culture, and fermentation culture. pH value of the seed medium and the fermentation medium is 6-8.
9	US2016168554 AA	*Streptomyces koganciensis ATCC 31394* Enzyme: hyaluronidase	1. Inoculate a bacterial culture medium in a bioreactor with an inoculum of recombinant cells

			2. Fermentation process (pH 6.7-7.1) 3. Centrifugation and re-suspending 4. Purification
10	US2015031085 AA	*P. pastoris GS 115* cells Enzyme: hyaluronidase	A method of overexpressing the leech hyaluronidase and a method of producing low-molecular-weight HA using the leech HAase. (See the abstract of the document for details). The reaction mixture is preferred to be incubated at pH 5.5, 38 °C for 4-8 hours.
11	CN101507733 A	Hyaluronidase	The degradation reaction conditions are: • pH 4～7, • Hyaluronic acid concentration 0.1～6%, • 20～70 ℃ of hydrolysis temperatures, • hydrolysis time 1～5 hour.
12	CN106309471 A	Recombinant hyaluronidase produced by *Saccharomyces*, recombinant hyaluronidase produced by plants, recombinant hyaluronidase produced by bacteria, and hyaluronidase extracted from animals	By using yeast hyaluronidase, hyaluronic acid fragments of 5-10 KD, 10-100 KD and 10-250 KD are obtained. The hyaluronic acid obtained is mixed in a 1: 1: 1 ratio.
13	WO21120521 A1	Hyaluronidase (yeast)	The hyaluronidase relative to the reaction solution is 1×10^4-1×10^5U/mL, the concentration of the macromolecular hyaluronic acid raw material is 40～200g/L,

			the reaction solvent is purified water, the enzymolysis time is 12～36h, the enzymolysis temperature is 35～45℃, and the stirring speed is 100～700 rpm.
14	JP1115903 A2	Protease in the presence of two or more enzymes (e.g., neutrase, papain, trypsin)	The starting material (with hyaluronic acid) is rapidly freeze and, without thawing, chopped using, e.g., a meat chopper followed by making the product into a paste using a homogenizer or ultrafine grinder and then performing sterilization at 70-140 ℃ for 1-60 seconds.
15	JP2006271351 A2	One or mixture of two or more kinds of *Aspergillus oryzae*, yeast, citric acid bacteria, lactic bacteria, acetic acid bacteria, and the *Aspergillus oryzae* or the like containing a fiber-splitting enzyme is added to the sterilized mixture and fermented.	One or a mixture of two or more kinds of organic acids having carboxy groups such as citric acid, lactic acid, tartaric acid and malic acid is added to the fermented hyaluronic acid, and the resultant mixture is matured by holding the mixture at a prescribed temperature. Tª: 35 °C to 45 °C
16	CN108220364 A	*Bacillus sp. A50 CGMCC NO.5744.* Enzyme: hyaluronidase	Conditions: 20 ~ 48 °C, pH 4 ~ 9.
17	CN108410928 A	Hyaluronidase	1. Add hyaluronidase 2. Redissolve the hyaluronic acid with the enzymolysis liquid 3. Filter and spray dried the mixture pH of the reaction system is 3.5-8.0. and temperature 25-50 ℃.

18	CN109517012B	Hyaluronidase	1. Enzymolysis of hyaluronic acid 2. Separate the hyaluronic acid oligosaccharide 3. Desalting step of hyaluronic acid oligosaccharide
19	CN102876748B	*Bacillus sp. A50 CGMCC NO.5744* Enzyme: hyaluronidase	1. Preparing the hyaluronic acid or the salt 2. Enzymolysis (the temperature of solution be that 20 - 48 ℃, pH is 4 - 9) 3. Inactivation 4. Filtering 5. Settling 6. Dewatering and drying.

Each of the inventions is summarized in more detail below, highlighting the microorganisms and enzymes that are involved in the processes, as well as the most important methods and conditions carried out.

1. US2011281817 AA

The document describes a method for producing hyaluronic using ***Streptococcus* cells**. These cells express the enzymes required for hyaluronic acid synthesis, wherein the activity or amount in the cells of one or more enzymes selected from **phosphoglucoisomerase, D-fructose-6-phosphate amidotransferase, phosphoglucosamine mutase, glucosamine-1-phosphate acetyl transferase, N-acetylglucosamine-1-phosphate pyrophosphorylase, glucosamine-6-phosphate acetyl transferase,** and **phosphoacetylglucosamine mutase** has been increased. On the other hand, the activity or amount in the cells of one or more enzymes selected from **UDP-N-acetylglucosamine 1-carboxyvinyltransferase** and **undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase** has been decreased or abrogated.

For the synthesis of hyaluronic acid by *Streptococcus* cells, one or more substrates selected from **UDP-N-acetylglucosamine, N-acetylglucosamine and glucosamine** are provided.

The hyaluronic acid produced using this method may have an average **molecular weight** of at least **3 or 3.5 MDa**. The hyaluronic acid may be substantially **non-crosslinked**.

The document also provides a **cosmetic composition** comprising the hyaluronic acid and a cosmetically acceptable carrier, excipient, or diluent.

Lotions according to the present invention include those suitable for application to the skin. Lotions for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturiser such as glycerol, or oil such as castor oil or arachis oil.

Creams, ointments or pastes according to the present invention are semi-solid formulations of hyaluronic acid for external application. They may be made by mixing hyaluronic acid in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with a greasy or non-greasy basis. The base may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap, a mucilage, an oil of natural origin such as almond, corn, arachis, castor or olive oil, wool fat or its derivatives, or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or macrogols.

Methods and Cells for Increasing Enzyme Expression or Activity

The specific enzymes identified as giving rise to an increase in HA MW are phosphoglucoisomerase (HasE, Pgi—EC 5.3.1.9), D-fructose-6-phosphate amidotransferase (GlmS—EC 2.6.1.16) and glucosamine-1-phosphate acetyl transferase/N-acetylglucosamine-1-phosphate pyrophosphorylase (HasD, GlmU—EC 2.3.1.4 and 2.7.7.23).

Increased expression/activity may be measured relative to an equivalent wild-type strain which has not been genetically modified, and which is grown under standard conditions (such as 37 °C. in rich media (M17G) or in chemically defined media (CDM) supplemented with 2% w/v D-glucose). For example, in the case of mucoid Group C *Streptococcus equi subsp. zooepidemicus*, a suitable control strain is ATCC 35246.

Increased activity of the enzymes is effected by genetically engineering the cells by introducing one or more nucleic acid sequences that direct expression of the enzymes. Such sequences can be introduced by various techniques, such as the introduction of plasmid DNA into cells using electroporation followed by subsequent selection of transformed cells on selective media. These heterologous nucleic acid sequences may be maintained extra chromosomally or may be introduced into the host cell genome by homologous recombination.

Methods and Cells for Increasing Substrate Levels

Elevated levels in streptococci of substrates involved in HA biosynthesis have been seen to lead to an increase in the molecular weight of the HA produced.

It has been further determined that enhanced levels of particular substrates such as glucosamine, N-acetylglucosamine and UDP-N-acetylglucosamine, and accordingly an increase in the molecular weight of the HA produced, may be achieved through a variety of methods. These methods include provision of additional amounts of the particular substrates or substrate precursors. This may be achieved, for example, by increasing endogenous production of the particular substrates or substrate precursors, or by exogenously increasing bioavailability of the particular substrates or substrate precursors. Other methods include downregulating or abrogating the activity or amount of enzymes that recruit these substrates or substrate precursors into different biosynthetic pathways, such as UDP-N-acetylglucosamine 1-carboxyvinyltransferase (UDP-NAG-CVT).

The document describes methods for producing HA by providing substrate precursors for UDP-NAG. These precursors may include glucosamine, N-acetylglucosamine and UDP-N-acetylglucosamine. Additionally, such methods further encompass providing metabolites including glutamine, acetyl-CoA and UTP.

These methods include transforming, transfecting or transducing HA-producing streptococcal cells with an expression vector encoding an enzyme producing said substrate or a precursor thereof. Introduction of the expression vector may be achieved by electroporation, followed by subsequent selection of transformed cells on selective media. Heterologous nucleic acid sequences thereby introduced into the cells may be maintained extrachromosomally or may be introduced into the host cell genome by homologous recombination.

Methods and Cells for Decreasing Enzyme Expression or Activity

Methods for downregulating or abrogating the activity or amount of an enzyme in a cell, such as UDP-N-acetylglucosamine 1-carboxyvinyltransferase (UDP-NAG-CVT) (murA) or MurG transferase (murG) (undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase), include disrupting the gene encoding the enzyme such that transcription of the gene is decreased or abrogated, for example, by “knocking out” the gene through insertional or deletional disruption, or through some other form of directed or random mutagenesis that targets either the gene or cofactor involved in transcription of the gene.

UDP-NAG-CVT typically exists in HA-producing streptococcal cells in two isoforms, each of which originate from separate genes. Accordingly, it has been determined that one gene encoding UDP-NAG-CVT may be downregulated or abrogated without compromising the viability of the streptococcal cells.

Other methods for downregulating or abrogating the activity or amount of an enzyme in a cell include disrupting translation of the mRNA transcribed from the gene, for example, through the use of antisense mRNA or interfering RNA, such siRNA. Further methods for downregulating or abrogating the activity or amount of an enzyme in a cell include targeting the enzyme with an antagonist such a small molecule or an antibody.

2. US2015175991 AA

The document describes a process for preparing **hyaluronidase** using a ***Bacillus sp.,*** which has a deposit access number of CGMCC NO. 5744.

This process comprises the following steps:

(1) Subjecting the bacillus to slant culture to obtain a slant strain.

(2) Inoculating the slant strain to a sterilized seed culture medium, and culturing under the conditions of 25 °C-40 °C, 100-200 rpm for 10-24 hours, to obtain a seed solution.

(3) Inoculating the seed solution to a sterilized fermentation culture medium, and culturing under the conditions of 25 °C-40 °C, 100-300 rpm for 12-24 hours, to obtain a hyaluronidase fermentation broth.

(4) Separating the fermentation broth by centrifugation to obtain a supernatant.

(5) Subjecting the supernatant to ammonium sulphate fractional precipitation, filtration (e.g., filtration using 0.65 μm microfiltration membrane), to obtain a crude hyaluronidase.

(6) Dissolving the crude hyaluronidase obtained in step (5) in a phosphate buffer solution, removing small molecular impurities by ultrafiltration, to obtain a purified hyaluronidase.

Optionally, step (6) can be carried out by the following steps (6-1) to (6-3):

(6-1): Dissolving the crude hyaluronidase of step (5) in a buffer solution with pH 4.5-8.0 to obtain a crude enzyme solution; loading the crude enzyme solution to a dialysis bag with a molecular cutoff of 3.0×10^{3}-1.4×10^{4} Da, placing in a buffer solution with pH 4.5-8.0, dialyzing at 4 °C overnight.

(6-2): Subjecting the dialyzed crude enzyme solution to ion exchange chromatography separation, in which chromatography column packing of DEAE agarose gel FF medium and 0-0.5M NaCl solution for gradient elution are used and collecting elution peaks.

(6-3): Subjecting the hyaluronidase sample obtained in step (6-2) to vacuum freeze drying to obtain white powder as hyaluronidase.

Hyaluronidase sequence is shown in SEQ ID NO: 1 (See Annex I).

The patent document also describes a **process for preparing oligomeric hyaluronic acid or oligomeric hyaluronate**, comprising the step of degrading hyaluronic acid or salts thereof with molecular weight greater than 10 kDa by:

1) Preparing the solution of hyaluronic acid or salts thereof: adding hyaluronic acid or salts thereof with molecular weight greater than 10 kDa to purified water, to obtain a solution with a concentration of 1% w/v-30% w/v. To per 1 kg of hyaluronic acid or salts thereof, 2×10^{7}-5×10^{7} IU of bacillus hyaluronidase is added. Hyaluronate is selected from the group consisting of sodium salt, potassium salt, magnesium salt, calcium salt and zinc salt of hyaluronic acid.

2) Enzymolysis: adjusting the temperature of the solution of step 1) to 20 °C-48 °C, pH to 4-9, then adding bacillus hyaluronidase to the solution, degrading the hyaluronic acid or salts thereof to a desired molecular weight, to obtain an enzymolysis solution. The temperature for enzymolysis is 35 °C-45 °C, the pH for enzymolysis is 5.5-7.5, and hyaluronic acid is enzymolyzed to a molecular weight of greater than or equal to 3000 Da. An acid or a base is used to adjust pH to 4-9. Said acid is selected from the group consisting of hydrochloric acid, glacial acetic acid, sulfuric acid and phosphoric acid, said base is sodium hydroxide or potassium hydroxide.

3) Inactivation: maintaining the enzymolysis solution at 50 °C-90 °C for 10-60 minutes, to inactivate the bacillus hyaluronidase.

4) Filtration: adding a soluble inorganic salt to the inactivated enzymolysis solution, stirring until it is completely dissolved, then filtering with 0.45 μm filtration membrane to obtain a filtrate, wherein to per 100 mL of enzymolysis solution, 0.1-10 g of the soluble inorganic salt is added. The soluble inorganic salt is selected from the group consisting of sodium salt, potassium salt, calcium salt, zinc salt and magnesium salt; preferably, is chloride, sulphate or nitrate of sodium, potassium, calcium, zinc or magnesium.

5) Precipitation: uniformly mixing the filtrate of step 4) with alcohol or ketone in 3-20 times volume of the filtrate, to precipitate oligomeric hyaluronate. The alcohol or ketone is ethanol, acetone, methanol, propanol, or isopropanol.

6) Dehydrating and drying: separating out the oligomeric hyaluronate precipitate of step 5), dehydrating with an organic solvent, then vacuum drying, to obtain oligomeric hyaluronate. The organic solvent used for dehydrating is ketone or alcohol.

The molecular weight is greater than or equal to 3000 Da, and less than 10^4 Da.

3. US2019224232 AA

The document describes a process for the preparation and purification of the sodium salt of HA from the fermentation broth of ***Streptococcus*** *(Streptococcus equi* sub specie equi 68222, mutant H-1.) or ***Bacillus*** or from rooster combs comprising the following steps:

1. Inactivation (for the purification of a HA produced from the fermentation of *Streptococcus* and *Bacillus*):

1.1. Acidification of the fermentation broth to a pH of 4-5; HCl 1N is preferably used.

1.2. Thermal treatment of the broth, under stirring (this treatment is not effected if a HA with a very high viscosity is produced).

1.3. Elimination of the biomass by means of filtration on pads of Celite (chemical name: silica dioxide; in an amount of from 30-40 g/litre), possible further filtration with filters having a filtration degree of 0.5 µm.

1.4. Neutralization to pH 6.5-7.5 with aqueous NaOH at 20%.

2. Extraction: in the case of homogenate from combs, the corresponding thermal treatment is first effected contemporaneously with its enzymatic digestion and subsequent filtration (to eliminate the undigested biological residue), followed by the following common phases:

2.1. Addition of Celite (in an amount of from 20 to 60 g/litre of broth/litre of enzymatic digest, i.e. per litre of medium containing non-purified HA) and complexing with Cetyl Pyridinium Chloride (CPC) (4-20 g/litre/litre of enzymatic digest, preferably 5-15 g/litre), under stirring, for at least 30 minutes and subsequent sedimentation for at least 30 minutes.

2.2. Elimination of the liquid phase.

2.3. Solubilization of the HA present in the solid phase in NaCl (a 0.3M aqueous solution is preferably used) under stirring for a period of 4 to 24 h, filtration by means of filtering cloths to separate the residual Celite and filters with a filtration degree of 3 µm (polypropylene filters are preferred) and collection of the first extract as sodium salt of HA; this procedure should be repeated from 1 to 4 times.

2.4. Joining the extracts.

2.5. Addition to the joined extracts of a resin of the aromatic type (in an amount of from 10 to 60 g/litre of extract) with a pore radius of 200-300 Angstrom (the resin DIAION HP20 (or HP20L)). This treatment is effected under stirring for at least 8 h.

2.6. At least a filtration by means of filtering cloths (preferably made of polypropylene) to separate the resins from the sodium salt of HA, and possibly at least a filtration with 3 μm-filtration degree filters (polypropylene filters).

3. Purification: in the case of the sodium salt of HA obtained from rooster combs, this step can be possibly preceded by the precipitation in ethanol of the sodium salt of HA obtained in the previous step, with the elimination of the above solvent and re-solubilization of the precipitate in purified water to restore the starting volume and subsequently proceeding with the following purification phases, regardless of the source selected:

3.1. Addition of NaOH (a 0.2-0.4 M solution is preferably used) in water, under stirring.

3.2. Neutralization to a pH ranging from 8 to 9, with HCl (at 37%).

3.3. Filtration, a filter with a filtration degree of 3 μm is preferable (polypropylene filters).

3.4. Precipitation and at least a washing of the sodium salt of HA sodium salt coming from step 3.3 with ethanol, final washing in an organic solvent (acetone).

3.5. Drying of the sodium salt of HA as known to skilled persons in the field, preferably from 25 to 40 °C for not less than 15 h, under vacuum.

After this, it can be subjected to a heat treatment of HA from *Streptococcus* or *Bacillus* fermentation broth, to achieve HA of different viscosity:

- 60 ± 5 °C for 5-40 minutes: this allows the production of a HA with a high viscosity (17-24 dl/g - 20 to 1450 kDa),
- 70 ± 5 °C for 5-40 minutes: this allows the production of a HA with a medium viscosity (10-15 dl/g - 450 to 780 kDa).
- 90 ± 5 °C for 150-300 minutes: this allows the production of a HA with a low viscosity (3-6 dl/g - 90 to 231 kDa),

If the thermal treatment is not effected, the final viscosity is equal to or over 29 dl/g (1885 kDa), therefore the purified product is a sodium salt of HA having a very high viscosity.

4. CN112501088 A

The document describes a method for producing **hyaluronidase** using ***Bacillus CQMU-D*** (*Bacillus sp.* CQMU-D). This method comprises the steps of:

1. Activating the preserved bacillus strain by using a slant culture medium to obtain a fresh strain. The slant culture medium comprises: 0.5 to 1.8 % of peptone, 0.5 to 1.8 % of yeast powder, 2 % of agar powder and the pH value of the slant culture medium is 6.5 to 7.5.

2. Inoculating the fresh strain obtained in step 1 into sterilized seed culture medium, and culturing for 16-24h in a shaker at 25-37 °C and rotation speed of 120. The seed culture medium comprises: 0.5-1.8% of peptone, 0.5-1.8% of yeast powder and 0.05-0.1% of K_2HPO_4, 0.01-0.05%$CaCl_2$, 0.05-0.1%$MgSO_4$. The pH of the seed culture medium is 6.5-7.5.

3. Inoculating the seed solution cultured in the step 2 into a sterilized fermentation medium, and culturing for 16-24h in a shaking table with the temperature of 25-37 °C and the rotation speed of 120. The fermentum medium comprises: 0.5-1.8% of peptone, 0.5-1.8% of yeast powder and 0.05-0.1% of K_2HPO_4, 0.01-0.05%$CaCl_2$, 0.05-0.1%$MgSO_4$, 0.05 mL of Tween 80, The pH of the fermentation medium is 6.5-7.5.

4. Centrifuging the hyaluronidase fermentation broth in the step 3 for 10-20 min.

5. Re-dissolving the crude protein collected in the step 4 by using a phosphate buffer solution and removing small molecular impurities by ultrafiltration or dialysis to obtain the purified hyaluronidase.

The hyaluronidase sequence is set forth in SEQ ID NO: 2 (See Annex I).

The document also protects the use of the enzyme in cosmetic products.

5. US2009312283 AA

The document describes a **branched hyaluronic acid**, wherein the linear backbone comprises hyaluronic acid in which one or more N-Acetyl-Glucosamine has been deacetylated to Glucosamine, with branching sidechain(s) covalently linked to the primary amine(s) of said deacetylated Glucosamine thus forming a secondary amine(s). The branching sidechain(s) comprises hyaluronic acid.

In this deacetylated hyaluronic acid (dHA), one or more N-Acetyl-Glucosamine has been deacetylated to Glucosamine by **chemical and/or enzymatic treatment**. It can be done by using hydrazine monohydrate together with hydrazine sulphate, or by using an **enzyme having HA deacetylase activity**.

The document also describes a method for producing a branched hyaluronic acid, comprising the steps of:

1. Providing a linear hyaluronic acid backbone, wherein one or more N-Acetyl-Glucosamine has been deacetylated to Glucosamine.

2. Reacting a biocompatible polymer comprising at least one free reducing aldehyde group with the primary amine(s) of the one or more Glucosamine of (a) by reductive N-alkylation; to form a branched hyaluronic acid.

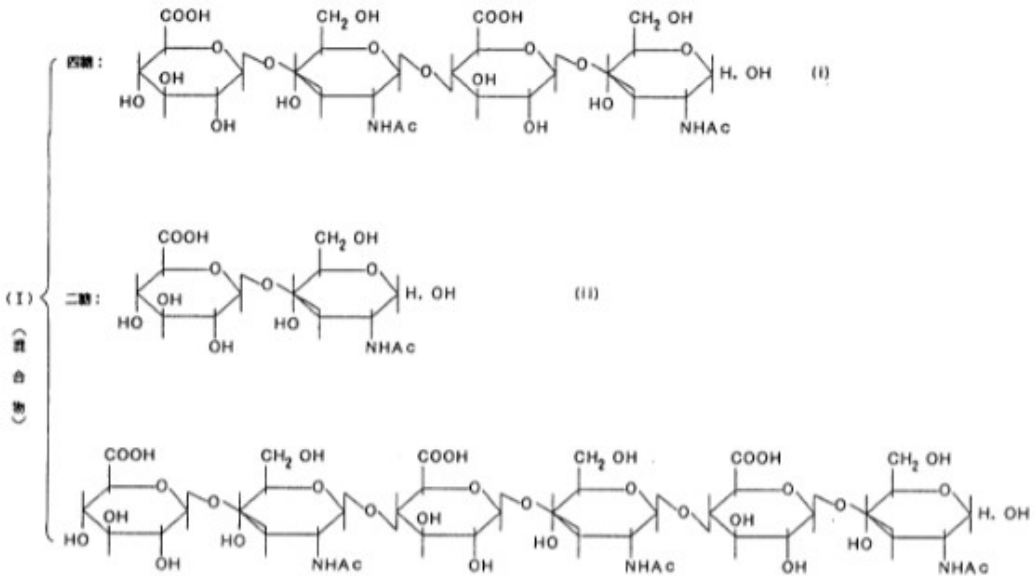
50% or less of the N-Acetyl-Glucosamines in the linear hyaluronic acid backbone have been deacetylated to Glucosamine.

The linear hyaluronic acid backbone has an average molecular weight in the range of 10-3.000 kDa.

The reductive N-alkylation reaction is done in the presence of sodium cyanoborohydride, $NaCNBH_3$.

6. JP1272511 A2

The document describes a cosmetic additive comprising oligosaccharides obtained by treating hyaluronic acid or a salt thereof with **testicular hyaluronidase** or **bacterial hyaluronidase**, namely tetrasaccharide shown by formula I, disaccharide shown by formula II, hexasaccharide shown by formula III and unsaturated disaccharide shown by formula IV or a mixture thereof or a cleft unsaturated disaccharide shown by formula V obtained by reducing the unsaturated disaccharide shown by the formula IV (**Figure 1**). Especially a combination of the tetrasaccharide, the disaccharide and the hexasaccharide, the unsaturated disaccharide alone and the cleft unsaturated disaccharide alone are preferable, and the amount of the additive added to the cosmetic base is 0.5-2wt.%. The compound has high stability to pH, light and heat, extremely excellent shelf stability and is not limited by cosmetic bases at all.



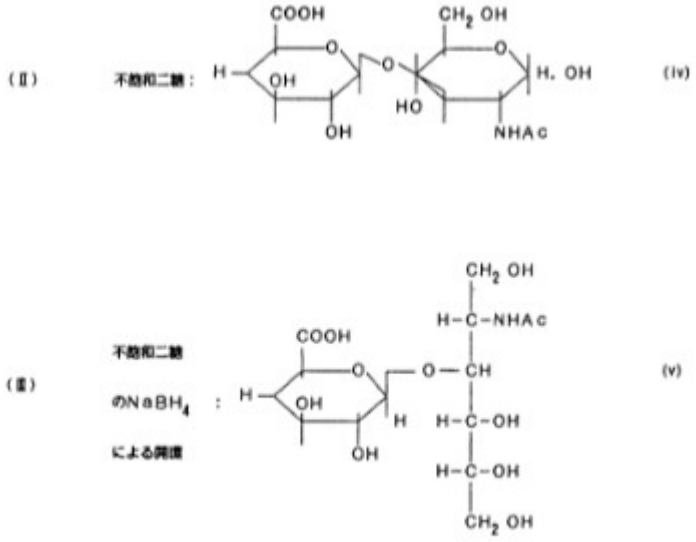


Figure 1. Different oligosaccharides obtained in JP1272511 A2

7. CN110331178 A

This document describes a low molecular weight hyaluronic acid production method using **hyaluronidase** from ***Arthrobacter globiformis*** (SEQ ID NO: 3 – See Annex I). This method comprises the steps:

1) Preparation of hyaluronic acid or its salting liquid: add hyaluronic acid or its salt (molecular weight is greater than 600 kDa) into purified water.

2) Digestion: the temperature of hyaluronic acid or its salting liquid is 20-50 °C, the pH is 5-10, 100U / g-5 × 10 is added in the solution that becomes hyaluronic acid or its salt. Hyaluronidase resolves (379Da-600kDa). Enzyme liquid is obtained. Adjust the temperature 20-45 °C and the pH 5.5-8.

3) Inactivation: the enzymolysis liquid is kept for 5-30 minutes at 55-100 °C and the enzyme is inactivated. Then the temperature is adjusted to 60-80 °C.

4) Filtration: add 0.01-2 mol/L of solubility inorganic salts to enzymolysis liquid, stir to completely molten, filtering and obtains filtrate. The inorganic salts are at least one of sodium salt, sylvite, calcium salt, magnesium salts or zinc salt.

5) Precipitation: add slowly alcohol or ketone into the filtrate of step 4), and uniformly mix. The alcohol or ketone are at least one of methanol, ethyl alcohol, propyl alcohol, butanol or acetone.

6) Dehydrate: collection of small molecular weight hyaluronic acid or its precipitating salt.

The molecular weight ranges of micromolecule hyaluronic acid or its salt are **379Da-600kDa**.

This micromolecule of hyaluronic acid or its saline preparation improves skin hydration, anti-aging, application in antioxidants, anti-inflammatories in food, cosmetics, or drugs.

8. US2021198620 AA

The document describes an *Enterobacter* that degrades hyaluronic acid. The ***Enterobacter sp.*** CGJ001 accession Number CGMCC NO. 18661 can degrade hyaluronic acid.

The document also describes a method of producing **hyaluronidase** comprising subjecting the *Enterobacter CGJ001* to plate culture, seed culture, and fermentation culture:

(1) Plate culturing the *Enterobacter CGJ001* to obtain plate strains.

(2) Inoculating the plate strains into sterilized seed culture medium and incubating at 30-40 °C and 150-300 rpm for 12-24 h to obtain seed liquid.

(3) Inoculating the seed liquid into a sterilized fermentation medium and incubating at 30-40 °C and 150-300 rpm for 12-24 h to obtain a hyaluronidase-containing bacterial liquid.

The seed medium and fermentation medium components comprise 1-10 g/L hyaluronic acid, 1-5 g/L K_3PO_4, 0.1-1 g/L $MgSO_4$, 1-10 g/L peptone, 1-10 g/L yeast powder.

The degradation product of hyaluronidase by the enzyme is 2-6 sugars.

The *Enterobacter CGJ001* has excellent enzyme activity, strong specificity for hyaluronic acid, good thermal and pH stability, and is suitable for scale-up production, thereby replacing hyaluronidase extracted from expensive animal tissues. It has a broad application prospect in the fields of medicine and cosmetics.

9. US2016168554 AA

The document describes a method to produce **hyaluronidase** from ***Streptomyces koganciensis ATCC 31394*** comprising the following steps:

1. Inoculating a bacterial culture medium in a bioreactor with an inoculum of recombinant cells that contain at least one vector comprising the sequence of cDNA encoding the hyaluronidase from *S. koganeiensis*. The recombinant cell is selected from a cell of *Escherichia coli* and one of *Bacillus subtilis.*

2. Subjecting the content of the bioreactor to fermentation at a pH between 6.7 and 7.1 in the presence of a glycerol solution.

3. Adding an inducer of the *lac* genes to the mixture of step 2.

4. Subjecting the mixture to an induction period of between 8 and 24 hours.

5. Centrifuging the bacterial cells obtained in step 4.

6. Re-suspending the pellets and subjecting the resulting suspension to osmotic shock.

7. Extracting the periplasmic proteins by centrifugation of the suspension.

8. Purifying the protein fraction having hyaluronidase enzymatic activity by a sequence of:

i. strong ion-exchange chromatography and isolation of the hyaluronidase enzymatic activity fraction.

ii. weak cation-exchange chromatography and isolation of the hyaluronidase enzymatic activity fraction.

iii. aromatic hydrophobic interaction chromatography and isolation of the hyaluronidase enzymatic activity fraction.

Hyaluronidase from *Streptomyces koganeiensis* in purified form is free from cysteine and has an endotoxin content<0.5 U/mg. This hyaluronidase can be used for **cosmetic applications** and/or to improve the aesthetic appearance.

10. US2015031085 AA

The document describes an isolated **leech hyaluronidase** (HAase), wherein the nucleotide sequence encoding said HAase comprises a member selected from the group consisting of:

a) SEQ ID NO: 4 (See Annex I).

b) A nucleotide sequence with one or several nucleotides substituted, deleted, or added based on SEQ ID NO: 4.

c) A nucleotide sequence having 85% identity with SEQ ID NO: 4.

d) A nucleotide sequence encoding a polypeptide of SEQ ID NO: 5 (See Annex I):

e) A nucleotide sequence with one or several nucleotides substituted, deleted, or added based on a nucleotide sequence of d).

f) A nucleotide sequence having 85% identity with a sequence of d).

The document also describes a **method of overexpressing the leech hyaluronidase**:

1. Fusing 6 His tags to the N-terminus of said HAase to obtain a His-HAase gene.

2. His-HAase gene is ligated with plasmid pPIK9K to create a His-HAase pPIK9K plasmid.

3. Transforming said His-HAase plasmid into a host cell (His-HAase pPIK9K plasmid is transformed into *P. pastoris GS 115*).

4. Expressing said His-HAase gene in said host cells. The expression of said His-HAase gene comprising the steps of:

a) cultivating a seed culture of *P. pastoris GS 115* cells carrying said His-HAase in YPD medium at 30 °C.

b) transferring the seed culture into BMGY medium and incubating at 30 °C, 200 rpm until the OD_{600} of the *P. pastoris GS 115* culture reaches 4-6.

c) transferring the cells to BMMY medium and cultivating at 30 °C, 200 rpm, adding 1% (v/v) methanol every 24 hours.

d) filtering the culture supernatant containing HAase through a 0.45 μm filter membrane and loading the filtrate onto a gravity-flow column filled with Ni-NTA agarose.

e) washing the column with a stepwise gradient of imidazole in a phosphate buffer.

f) eluting the bound His-HAase protein from the column with a phosphate buffer containing 500 mM imidazole, and dialyzing the eluent to remove imidazole and salts, and obtain pure His-HAase protein.

The document also describes a method of producing low-molecular-weight HA using the leech HAase. The reaction mixture which contained high-molecular-weight HA (Molecular weight 10^4-10^7 kDa) and pure leech HAase (100-13000 U/mg HA) is incubated at pH 4.0-8.0, 10 °C-65 °C for 4-8 hours. Before the reaction, the high-molecular-weight HA is prepared to have a concentration of 1-100 g/L in 50 mM citrate buffer (pH 5.5). The leech HAase is dissolved in water to make an enzyme solution. The reaction mixture is preferred to be incubated at pH 5.5, 38 °C for 4-8 hours.

- To produce decasaccharide (HA10) and octasaccharide (HA8), 0.8 mL high-molecular-weight HA (2 g/L), 8 μL HAase (2.43×105 U/mL) and appropriate amount of citrate buffer (pH 5.5) are mixed to form 1 mL reaction system. The mixture is incubated at 38 °C for 4 hours to generate HA10 and HA8.
- To produce tetrasaccharide (HA4) and hexasaccharide (HA6), 0.8 mL high-molecular-weight HA (2 g/L), 41 μL HAase (2.43×105 U/mL) and appropriate amount of citrate buffer (pH 5.5) are mixed to form 1 mL reaction system. The mixture is incubated at 38 °C for 8 hours to generate HA4 and HA6.
- To produce tetrasaccharide (HA4), hexasaccharide (HA6) and octasaccharide (HA8), 0.8 mL high-molecular-weight HA (2 g/L), 8 μL HAase (2.43×105 U/mL) and appropriate amount of citrate buffer (pH 5.5) are mixed to form 1 mL reaction system. The mixture is incubated at 38 °C for 6 hours to generate HA4, HA6 and HA8.
- To produce tetrasaccharide (HA4), hexasaccharide (HA6), octasaccharide (HA8) and decasaccharide (HA10), 0.8 mL high-molecular-weight HA (2 g/L), 10 μL HAase (2.43×105 U/mL) and appropriate amount of citrate buffer (pH 5.5) are mixed to form 1 mL reaction system. The mixture is incubated at 38 °C for 5 hours to generate HA4, HA6, HA8 and HA10.

11. CN101507733 A

The document describes a hyaluronic acid nano-micromolecule characterized in that its average number of molecules is less than 1000 Daltons and the content of hyaluronic acid in the form of a dimer, trimer or tetramer exceeds 90%.

The document also provides a method of preparing hyaluronic acid from nano micromolecule. The reaction medium used is water, an organic solvent or oxidant cannot be used.

First hydrolysis in acid medium: pH 1.5 - 4, hyaluronic acid concentration 0.1 ～ 6%, hydrolysis temperature 70 ～ 95 °C, hydrolysis time is 1 ～ 5 hours.

Enzymatic degradation: the enzyme used for enzymatic degradation is a **hyaluronidase**, and the degradation reaction condition is **pH value 4 ～ 7**, hyaluronic acid concentration 0.1 ～ 6%, **20 ～ 70 ℃** hydrolysis temperature and the hydrolysis time is 1 to 5 hours.

The hyaluronic acid obtained improves the immunity capacity, can preserve the moisture of the whole skin, softens, reduces skin xerosis, improves skin elasticity, relieves wrinkles with a remarkable effect.

12. CN106309471 A

The document describes a method for obtaining low molecular weight hyaluronic acid, between 5 and 250 KDa. The low molecular weight hyaluronic acid must not have bacterial endotoxin contamination.

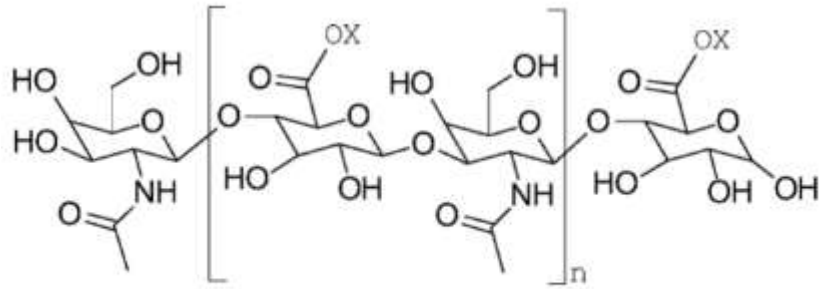
For this, **hyaluronidase** is used, which can be one or more of the following enzymes: recombinant hyaluronidase produced by Saccharomycetes, recombinant hyaluronidase produced by plants, recombinant hyaluronidase produced by bacteria, and hyaluronidase extracted from animals

By using **yeast hyaluronidase**, hyaluronic acid fragments of 5-10 KDa, 10-100 KDa and 10-250 KDa are obtained. The hyaluronic acid obtained is mixed in a 1: 1: 1 ratio.

This hyaluronic acid is used for preparing cosmetics or skin care products.

13. WO21120521 A1

The document describes an ultra-low molecular weight hyaluronic acid, characterized in that: the average molecular weight is less than **1200 Daltons**. The molecular weight distribution range is narrow, and it is a mixture of hyaluronic acid disaccharide to dodecasaccharide, The content of hyaluronic acid disaccharide is 5-40%, the content of hyaluronic acid tetrasaccharide is 40-70%, the content of hyaluronic acid hexasaccharide is 10-30%, and the content of hyaluronic acid octasaccharide is 1- 15% and the hyaluronic acid decasaccharide content accounts for 1-10%. The general structural formula of the ultra-low molecular weight hyaluronic acid is shown in the following formula I (**Figure 2**):



Formula I: n=0-5, X=H, K or Na.

Figure 2. General structural formula of the hyaluronic acid obtained in WO21120521 A1.

The molecular weight of the hyaluronic acid is **800-1000 Da**.

The document also describes **the method for preparing ultra-low molecular weight hyaluronic acid**, characterized in that: the macromolecular hyaluronic acid raw material is enzymatically hydrolyzed by **hyaluronidase** to obtain an average molecular weight of less than 1200 Daltons.

The hyaluronidase is a **leech-type hyaluronidase**, which is obtained by optimized expression of **yeast**. The operating conditions of the enzymatic hydrolysis reaction are that the added amount of the hyaluronidase relative to the reaction solution is 1x10^4 -1× 10^5 U/mL, the concentration of the macromolecular hyaluronic acid raw material is 40～200g/L, the reaction solvent is purified water, the enzymolysis time is 12～36h, the enzymolysis temperature is **35～45℃**, and the stirring speed is 100～700 rpm , the **pH** of enzymatic hydrolysis is **4.0-6.0**.

The obtained ultra-low-molecular-weight hyaluronic acid has better skin permeability and promotes damaged skin repair.

14. JP1115903 A2

The document describes an aqueous dispersion of the raw material containing large quantities of hyaluronic acid such as cockscombs, skins or synovial fluid which is treated with a **protease** in the presence of two or more enzymes (e.g., **neutrase, papain, trypsin**) followed by extracting the objective hyaluronic acid. Preferably, said raw material is rapidly freezed and, without thawing, chopped using, e.g., a meat chopper followed by making the product into a paste using a homogenizer or ultrafine grinder and then performing sterilization at 70-140 ℃ for 1-60 seconds.

15. JP2006271351 A2

The document describes a method involves carrying out malt/organic acid fermentation of a raw material hyaluronic acid containing a mineral. Concretely, the mineral is added to the raw material hyaluronic acid having about 500,000-1,200,000 molecular weight and the resultant mixture is sterilized by steaming with a prescribed amount of purified water. One or mixture of two or more kinds of ***Aspergillus oryzae*, yeast, citric acid bacteria, lactic bacteria, acetic acid bacteria, and the *Aspergillus oryzae*** or the like containing a **fiber-splitting enzyme** is added to the sterilized mixture and it is fermented. One or a mixture of two or more kinds of organic acids having carboxy groups such as citric acid, lactic acid, tartaric acid and malic acid is added to the fermented hyaluronic acid, and the resultant mixture is matured by holding the mixture at a prescribed temperature. The resultant product hyaluronic acid is filtered and extracted. The low-molecular weight hyaluronic acid having about **2,000 Da** molecular weight is efficiently produced by this method.

16. CN108220364 A

This document describes a kind of solid-liquid double-phase enzymolysis to prepare an ultra-low molecular weight hyaluronic acid oligosaccharide. This method includes the following steps:

（1）Buffer solution is digested to prepare the enzymolysis buffer solution. 20 ~ 48 °C, pH 4 ~ 9. Then adds the *Bacillus* hyaluronidase（*Bacillus* sp. *A50 CGMCC NO.5744*).

（2）Solid-liquid double-phase digestion：Zirconium oxide dispersion pearl is added into enzymolysis buffer solution under stirring, then rapidly joins high molecular weight hyaluronic acid or its salt solid to its mass-volume concentration be 5 ~ 25%. It digests under stirring to after being changed into the single-phase enzymatic hydrolysis system of liquid-liquid to solid-liquid double-phase enzymatic hydrolysis system.

（3）Ultrafiltration： enzyme digestion reaction liquid relates to ultrafiltration system. It obtains weight ≤3 kDa of the hyaluronic acid or its salt in enzyme digestion reaction liquid.

(4) Post – processing: filtering gained filtrate are spray-dried and obtain the ultra-low molecular weight hyaluronic acid oligosaccharide and its salt of molecular weight ≤ 3kDa.

17. CN108410928 A

This document describes a high-efficiency method for producing micromolecule hyaluronic acid dry powder. Steps:

(1) High molecular weight hyaluronic acid is dissolved in the reaction system, and the **hyaluronidase** solution is added (0.5-3 hours)

(2) High molecular weight hyaluronic acid is redissolved in the enzymolysis liquid. Enzyme solutions react for a certain time (1-10 hours).

(3) To obtain micromolecule with a high concentration of hyaluronic acid, the mixture is filtered and spray dried.

The pH of the reaction system is 3.5-8.0. and temperature 25-50 ℃.

The high concentration hyaluronic acid micromolecule and its dry powder have a molecular weight of 1-10 KDa.

The micro-molecule of dry powder hyaluronic acid is easily soluble in water, whereby a uniform, transparent and colourless liquid is obtained.

The hyaluronic acid micromolecule and its dry powder can be used in cosmetics, eye drops, cutaneous application on the skin and soft tissue operation.

18. CN109517012B

The document describes a method for preparing high-purity hyaluronic acid oligosaccharide comprises the following steps:

1. Enzymolysis of hyaluronic acid: hydrolyze hyaluronic acid by **hyaluronidase** to obtain a hyaluronic acid oligosaccharide mixture.

2. Separating hyaluronic acid oligosaccharide by using an ion exchange chromatographic column, and sequentially collecting components according to peak time. The ion exchange chromatographic column is used under the pressure of 0-5000 psi, the stationary phase of the ion exchange chromatographic column is a silica gel stationary phase bonded with quaternary ammonium strong anion exchange groups, and the ions are adsorbed on the silica gel stationary phase.

The ion exchange chromatographic column is Sepax HP-SAX, and the mobile phase used by the ion exchange chromatographic column is 0-0.5mol/L NaCl or Na_2SO_4.

The method of the present invention can provide a wider variety of o-HA (HA 4-HA 20).

19. CN102876748B

The invention discloses a method for preparing oligomeric hyaluronate by a digestion and the application thereof. ***Bacillus*** **hyaluronidase** obtained through fermented cultivation of ***Bacillus sp. A50 CGMCC NO.5744*** is used for degrading hyaluronic acid or a salt thereof, and the method comprises the steps of: preparing the hyaluronic acid or the salt thereof, enzymolysis, inactivation, filtering, settling, dewatering and drying.

1.Prepare hyaluronic acid or its salts solution: add in purified water the hyaluronic acid or its salts (10kDa).

2. Enzymolysis: the temperature of the solution is 20 ~ 48 ℃ and pH are 4 ~ 9.

3. Deactivation: enzymolysis solution is kept to 10 ~ 60 min at 50 ~ 90 ℃.

4. Filtration: in the enzymolysis solution after deactivation, adding processable inorganic salt, be stirred to completely and dissolve, then filtrate (0.45 μm with aperture membrane), obtains filtrate, adds the processable inorganic salt of 0.1 ~ 10g in every 100 mL enzymolysis solution.

5. Precipitation: add alcohol o ketone.

6. Dehydration: with dehydration of organic solvent, vacuum-drying and obtains oligomerization hyaluronate.

According to the method, the hyaluronidase produced by the *Bacillus* is used for degrading the hyaluronic acid or the salt thereof. The method is simple to operate and mild in condition, has no destroy on the product structure and is eco-friendly. The hyaluronidase for fermentation is low in cost and is suitable for large-scale industrial production. The prepared oligomeric hyaluronate has the advantages of good percutaneous absorption capability, high purity, no cytotoxicity, strong oxidation resistance and can be used in fields such as cosmetics, foods and medicines.

3. Scientific literature

Table 2 shows a summary of the organisms and enzymes described in each scientific document, as well as the most important methods and conditions.

Table 2. Organisms, enzymes, methods, and conditions of each scientific document.

	REFERENCE (TITLE)	ORGANISM / ENZYME	METHODS / CONDITIONS
1	Biotechnological production of hyaluronic acid: a mini review	*Streptococci, Bacillus, Agrobacterium, E. coli* and *Lactococcus.* Class II HAS enzyme	Bacterial production of HA and cell-free production of HA (*in vitro* system)

2	Key Factors for a One-Pot Enzyme Cascade Synthesis of High Molecular Weight Hyaluronic Acid	Enzymes AtGlcAK, AtUSP (both *Arabidopsis thaliana*), and PmPpA (*Pasteurella multocida*), BlNahK (*Bifidobacterium longum*), SzGlmU (*Streptococcus zooepidemicus*) and PmHAS (*P. multocida*)	The recombinant enzymes were produced in *E. coli* BL21 (DE3) and purified by immobilized metal affinity chromatography (IMAC).
3	Photosynthetic conversion of CO2 to hyaluronic acid by engineered strains of the cyanobacterium *Synechococcus sp. PCC 7002*	*Synechococcus sp. PCC 7002* HA synthases from *Pasteurella multocida* and *Streptococcus equisimilis*	Introducing HA synthases from *Pasteurella multocida* and *Streptococcus equisimilis* in cyanobacterium *Synechococcus sp. PCC 7002*
4	Enhanced Biosynthesis of Hyaluronic Acid Using Engineered *Corynebacterium glutamicum* Via Metabolic Pathway Regulation	*Corynebacterium glutamicum* Five enzymes (HasA-HasE)	Eight diverse operon combinations, including HasA, HasAB, HasAC, HasAD, HasAE, HasABC, HasABD, and HasABE. Batch and fed-batch cultures of *C. glutamicum/Δldh-AB* are performed in a 5-L fermenter.
5	Characterization of UDP-glucose dehydrogenase from *Pasteurella multocida* CVCC 408 and its application in hyaluronic acid biosynthesis	UDP-glucose dehydrogenase from *Pasteurella multocida CVCC 408*	A UGDH (PmuHasB, 45.9 kDa) from *Pasteurella multocida CVCC 408* was expressed in *Escherichia coli* BL21 (DE3). It was purified by two chromatographic columns with a specific activity of 6.58 IU/mg. The optimum pH and temperature were

			determined to be 10.0 and 37° C, respectively.
6	Magnetic macroporous bead cellulose functionalised with recombinant hyaluronan lyase for controllable hyaluronan fragmentation	*Streptococcus pneumoniae* hyaluronan lyase (SpnHL)	Production and characterization biocompatible magnetic macroporous bead cellulose (MBC) functionalised with *Streptococcus pneumoniae* hyaluronan lyase (SpnHL)
7	Large-scale Preparation, Purification, and Characterization of Hyaluronan Oligosaccharides From 4-mers to 52-mers	Testicular hyaluronidase	Hyaluronan (HA) was depolymerized by partial digestion with testicular hyaluronidase and separated into size-uniform HA oligosaccharides from 4-mers to 52-mers by anion exchange chromatography after removal of the hyaluronidase.
8	Novel Methods for the Preparation and Characterization of Hyaluronan Oligosaccharides of Defined Length	Testicular hyaluronidase	Methods for the purification of hyaluronan oligomers of defined size using size exclusion and anion-exchange chromatography following digestion of hyaluronan with testicular hyaluronidase. Digestion conditions: pH 5.2 and Tª 37 °C)
9	Enzymatic Production of Specifically Distributed Hyaluronan Oligosaccharides	Recombinant leech hyaluronidase (HAase)	High-molecular-mass hyaluronan (HA) was controllably depolymerized in pure

			aqueous solution with recombinant leech hyaluronidase. By modulating the concentrations of HAase and controlling the hydrolysis time, any molar-mass-defined HA oligomers could be efficiently produced.
10	High-yield novel leech hyaluronidase to expedite the preparation of specific hyaluronan oligomers	Leech hyaluronidases (H6LHyal)	H6LHyal exhibits superior enzymatic properties than the acidic BTH14 (a near-neutral optimum pH of 6.5 and an optimum temperature of 45 °C, with excellent temperature resistance).
11	Construction of saturated odd- and even-numbered hyaluronan oligosaccharide building block library	Bovine testicular hyaluronidase (BTH) and leech hyaluronidase (LHase)	A dynamic model for LHase hydrolysis was established, providing optimal preparation conditions for HA2nNA (n = 1–5). Then HA2nNA (n = 2–5) was further degraded by BTH to produce odd-numbered o-HAs.
12	Expression of a novel hyaluronidase from *Streptococcus zooepidemicus* in *Escherichia coli* and its application for the preparation of HA oligosaccharides	Novel hyaluronidase from *Streptococcus zooepidemicus*	The optimum enzymatic activity was achieved at 37 °C and p H 5.5.
13	Preparation and extensive characterization of hyaluronan with narrow molecular weight distribution	Enzymatic or chemical hydrolysis	The HA fractionation was performed using an anion-exchange chromatography and is applicable either after enzymatic or

			chemical hydrolysis of polymeric HA.
14	A novel hyaluronidase produced by *Bacillus sp. A50*	*Bacillus sp. A50* (hyaluronidase)	The optimal temperature and pH of HAase-B were 44 °C and pH 6.5, respectively. It was stable at pH 5–6 and at a temperature lower than 45 °C.
15	Characterization of Hyaluronan-Degrading Enzymes from Yeasts	HAases from two of the yeast isolates, *Pseudozyma aphidis* and *Cryptococcus laurentii*	The enzymes' pH and temperature optima were pH 3.0 and 37–45 °C (*P. aphidis*) and pH 6.0 and 37 °C (*C. laurentii*), respectively.
16	Hyaluronic acid of tailored molecular weight by enzymatic and acid depolymerization.	Hyaluronidase from bovine testes type I-S, chondroitinase from *Proteus vulgaris*	Enzymatic reactions were carried out in triplicate at 37 °C under magnetic stirring by adding the appropriate amounts of either enzyme to tubes containing 10 mL of HA solutions to reach enzyme to substrate ratios of 0.01, 0.025, 0.05, 0.075 and 0.01 units of CASE and 0.5, 1.0, 2.0, 7.0 and 12.0 units of HASE per mg of HA. pH 8 for reaction with CASE. pH 4 for HASE depolymerization.
17	Characterisation of separated end hyaluronan oligosaccharides from leech hyaluronidase and evaluation of angiogenesis.	Leech hyaluronidase (LHase)	LHase kept high activity when incubated at 38 ◦C for several hours. As a result, one gram HA was hydrolysed by a series of

			doses of LHase (4000–32,000 U/mL) in 100 mL distilled water with different incubation time at 38 ◦C, respectively

Each of the scientific documents is summarized in detail below, highlighting the microorganisms, enzymes involved in the processes, as well as the methods and conditions carried out.

1. Biotechnological production of hyaluronic acid: a mini review

In this review, they explore current knowledge of biosynthetic enzymes that produce HA, how these systems have been used commercially to produce HA and how the challenges of producing HA cheaply and safely are being addressed.

Currently, industrial production of HA is based on either HA extraction from animal tissues or via large-scale bacterial fermentation with genetically modified strains. These processes are widely used and have been able to generate HA products with molecular weights above 1 MDa (as half-life of the molecule will increase and persist longer while maintaining its physiological function), which is desirable for biomedical and cosmetic use.

Extraction from animal tissues

Extraction of HA has been widely carried out using other animal tissues including human umbilical cord, rooster comb, and bovine synovial fluid.

Today, although animal-derived HA still remains as an important resource for most HA-based products, alternative production processes have been sought for a number of reasons. First, the extraction processes have always experience technical limitations due to harsh extraction conditions that comes with grinding, acid treatment, and repeated extraction with organic solvents. A second problem is that animal HA may still be bound to cellular proteins including hyaluronidase, a HA-specific binding protein. Finally, extracting HA from animal tissues is costly as it takes considerable time to complete, is labour intensive and requires large facilities that can accommodate processes involved from collection of tissue from the animal to extraction and purification of HA.

Bacterial Production

In its early stage of development, Group A and C Streptococci that naturally produced HA were grown in fermenters and HA was purified. However, as these bacteria produce a number of toxins, alternative bacteria were sought. Once the genes that encode for the HA biosynthetic pathway were determined, several bacteria (*Bacillus, Agrobacterium, E. coli* and *Lactococcus*) were genetically modified to express these genes and produce HA.

The ***Bacillus*** production system (*B. subtilis*) is a well-characterized Gram-positive microorganisms (along with Group A and C Streptococci) established as industrial workhorses for the production of various products, including HA (Novozymes). The expression constructs utilizing *hasA* gene from *S. equisimilis* in combination with overexpression of

one or more of the three native *B. subtilis* precursor genes (homologous to *hasB, hasC* and *hasD* in Streptococci) were used. *B. subtilis* has highly developed biosynthetic capacity and capability to grow in industrial fermenters.

While production of HA is commonly observed in Gram-positive bacteria, many Gram-negative bacteria, such as ***E. coli*** do not produce HA, as they either lack key enzymes of the biosynthetic pathway, or express components of the pathway at very low levels. Most strains of *E. coli* commonly used in laboratories, such as JM109, do not synthesise HA for these reasons. If, however, UDP-glucose dehydrogenase (*kfiD*) from *E. coli K5* and HAS (*pmHAS*) from *P. multocida*, are expressed, it is possible to synthesise HA in *E. coli* strains such as JM109.

Cell-free production of HA (in vitro system)

The **Class II HAS from *P. multocida*** is amenable for cell free HA production as the enzyme is a peripheral (not an integral membrane protein) and does not need to be bound to the membrane in order to function. Furthermore, deletion studies have shown that removal of the membrane domains (residues 704–972) generates a soluble enzyme (known as pmHAS1-703) that retains the ability to synthesise HA. Using this cell-free system it is possible to produce HA with high molecular weight (~1–2 MDa) with low polydispersity.

2. Key Factors for a One-Pot Enzyme Cascade Synthesis of High Molecular Weight Hyaluronic Acid

The one-pot synthesis contains **three enzyme modules** (EM): UDP–GlcA-, UDP–GlcNAc-, and hyaluronan-module. In the EM UDP–GlcA and EM UDP–GlcNAc, AtGlcAK and BlNahK phosphorylate the monosaccharides GlcA and GlcNAc, respectively, to the corresponding sugar-1-phosphates with ATP consumption. AtUSP and SzGlmU convert them to UDP–GlcA and UDP–GlcNAc, respectively, using uridine triphosphate (UTP). PmPpA hydrolyzes the AtUSP and SzGlmU inhibiting by product pyrophosphate (PPi). Finally, PmHAS utilizes the nucleotide sugars for HA chain polymerization.

The one-pot synthesis is accomplished by three enzyme modules (EM): UDP–GlcA, UDP–GlcNAc, and hyaluronan module. The EM UDP–GlcA contains the enzymes AtGlcAK, AtUSP (both *Arabidopsis thaliana*), and PmPpA (*Pasteurella multocida*). The EM UDP–GlcNAc contains the enzymes BlNahK (*Bifidobacterium longum*), SzGlmU (*Streptococcus zooepidemicus*), and PmPpA. In the hyaluronan module PmHAS (*P. multocida*) polymerizes the HA chain using UDP–GlcA and UDP–GlcNAc as donor substrates (**Figure 3**).

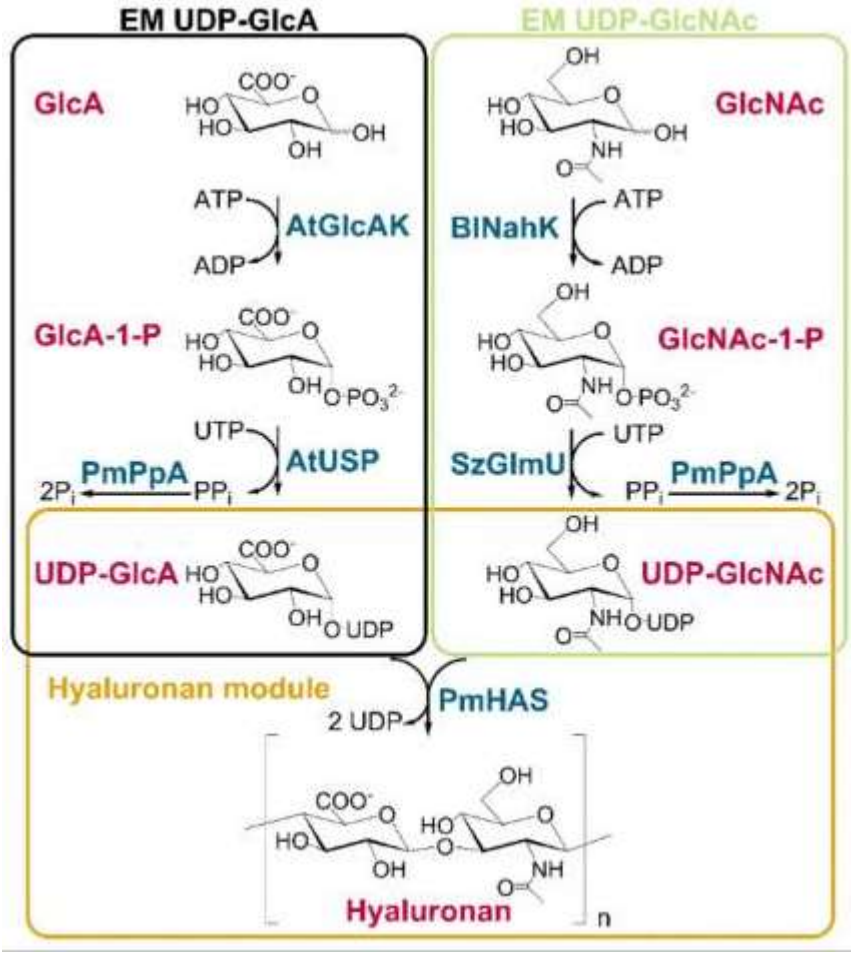


Figure 3. One-pot synthesis by a three-enzyme modules cascade of high molecular weight hyaluronic acid.

3. Photosynthetic conversion of CO_2 to hyaluronic acid by engineered strains of the cyanobacterium *Synechococcus sp. PCC 7002*

Hyaluronic acid (HA), consisting of alternating N-acetylglucosamine and glucuronic acid units, is a natural polymer with diverse cosmetic and medical applications. Currently, HA is produced by overexpressing HA synthases from gram-negative ***Pasteurella multocida*** (encoded by pmHAS) or gram-positive ***Streptococcus equisimilis*** (encoded by seHasA) in various heterotrophic microbial production platforms. Here they introduced these two different types of HA synthase into the fast-growing cyanobacterium ***Synechococcus sp. PCC 7002*** (Syn7002) to explore the capacity for producing HA in a photosynthetic system. Their results show that both HA synthases enable Syn7002 to produce HA photoautotrophically, but that overexpression of the soluble HA synthase (*PmHAS*) is less deleterious to cell growth and results in higher production. Genetic disruption of the competing cellulose biosynthetic pathway increased the HA titer by over 5-fold (from 14 mg/L to 80 mg/L) and the relative proportion of HA with molecular mass greater than 2 MDa. Introduction of *glmS* and *glmU*, coding for enzymes involved in the biosynthesis of the precursor UDP-N-acetylglucosamine, in combination with partial glycogen depletion, allowed photosynthetic production of 112 mg/L of HA in 5 days, an 8-fold increase in comparison to the initial *PmHAS* expressing strain. Addition of *tuaD* and *gtaB* (coding for genes involved in UDP-glucuronic acid biosynthesis) also improved the HA yield, albeit to a lesser extent.

4. Enhanced Biosynthesis of Hyaluronic Acid Using Engineered *Corynebacterium glutamicum* Via Metabolic Pathway Regulation

To avoid potential pathogenicity caused by its native producer, *Streptococcus*, efforts have been made to create a recombinant host for HA production. In this work, a GRAS (generally recognized as safe) strain, ***Corynebacterium glutamicum***, is engineered for enhanced biosynthesis of HA via metabolic pathway regulation. Five enzymes (**HasA-HasE**) involved in the HA biosynthetic pathway are highlighted, and eight diverse operon combinations, including HasA, HasAB, HasAC, HasAD, HasAE, HasABC, HasABD, and HasABE, are compared. HasAB and HasABC are found to be optimal for HA biosynthesis in *C. glutamicum*. To meet the energy demand for HA synthesis, the metabolic pathway that produces lactate is blocked by knocking out the lactate dehydrogenase (*LDH*) gene using single crossover homologous recombination. Engineered *C. glutamicum/Δldh-AB* is superior and had a significantly higher HA titer than *C. glutamicum/Δldh-ABC*. Batch and fed-batch cultures of *C. glutamicum/Δldh-AB* are performed in a 5-L fermenter. Using glucose feeding, the maximum HA titer reached 21.6 g/L, more than three folds of that of the wild-type *Streptococcus.*

5. Characterization of UDP-glucose dehydrogenase from *Pasteurella multocida CVCC 408* and its application in hyaluronic acid biosynthesis

UDP-glucose dehydrogenase (UGDH, EC 1.1.1.22) is an essential enzyme for HA synthesis. In this study, a UGDH (PmuHasB, 45.9 kDa) from ***Pasteurella multocida CVCC 408*** was expressed in *Escherichia coli BL21* (DE3). It was purified by two chromatographic columns with a specific activity of 6.58 IU/mg. The optimum pH and temperature were determined to be 10.0 and 37 °C, respectively. The activity was stable across the pH range 6-10, and had a half-life of about 3 h at 45 °C. The estimated apparent Km values for UDP-glucose and NAD (+) were 0.11 and 0.069 mM, respectively. The results indicated that PmuHasB was an alkaline and mesophilic UGDH. PmuHasB and PmuHasA (HA synthase, HAS) were co-expressed in *E. coli BW25113* to obtain a HA high-producing strain pBPAB/BW25113. It produced about 2.39 g/L HA in shake flask by using the method of whole-cell catalysis. Investigation of the different UGDHs on HA synthesis revealed that intracellular UGDH activity and HA total yield of pBPAB/BW25113 (0.15 IU/mg and 5.4 g/L) were higher than from pBPASB/BW25113 (0.013 IU/mg and 2.8 g/L) and pBPAEB/BW25113 (0.010 IU/mg and 2.22 g/L). These results indicated that the activity and stability of UGDH plays a significant role in HA production and should prove useful for further genetic engineering research with a view to construct other glucuronic acid polysaccharide synthesis pathways.

6. Magnetic macroporous bead cellulose functionalised with recombinant hyaluronan lyase for controllable hyaluronan fragmentation

In this study authors produced and characterised biocompatible magnetic macroporous bead cellulose (MBC) functionalised with ***Streptococcus pneumoniae*** **hyaluronan lyase (SpnHL)** that are compatible with these requirements and optimised their reaction and storage conditions. Immobilisation of SpnHL on MBC via reductive amination or MBC with fixed iminodiacetic acid (MBC-IDA) via a His8-tag had minimal impact on its catalytic activity. The MBC-IDA-SpnHL

carrier showed excellent operational and storage stability, and both carriers enabled reproducible time-controlled fragmentation of highly viscous HMW HA solutions, yielding HA fragments of appropriate molecular weight.

7. Large-scale Preparation, Purification, and Characterization of Hyaluronan Oligosaccharides From 4-mers to 52-mers

HA oligosaccharides were isolated and purified from **testicular hyaluronidase** digests of ~200 g of rooster comb HA. Testicular hyaluronidase is an endo-GlcNAc hydrolase that generates a ladder of HA oligosaccharides that vary by one repeat disaccharide. The anion exchange procedure that was used successfully separated HA oligosaccharides with 2 to 26 repeats (4-mers to 52-mers) that were of high purity and free of protein, DNA, and endotoxin contaminants.

HA was partially degraded by bovine testicular hyaluronidase. To a solution containing 200 g of HA in 10 L of 100 mM phosphate buffer (pH 5.3) containing 150 mM sodium chloride, 2 MU hyaluronidase was added, and enzymatic digestion was performed at 37 °C for 6–40 h. The incubation time of the hyaluronidase varied according to the sizes of HA oligosaccharides to be obtained. The reaction was stopped by boiling for 20 min. The sample was centrifuged at 10,000 rpm for 30 min, and the supernatant was concentrated and lyophilized. The lyophilized sample was dissolved in distilled water, and each HA oligosaccharide was isolated from the parent digest by anion exchange chromatography on a Dowex 1 × 2 column.

The HPLC and FACE methods confirmed the purity in each HA oligosaccharide prepared in the present study. The molecular size of each, determined by ESI-MS, coincides with their theoretical calculated mass.

These data, and the negligible contents of endotoxins, DNA and protein, indicate that the HA oligosaccharides are pure, size-uniform for a wide variety of sizes. Thus, the procedures used, hyaluronidase digestion and anion exchange chromatography, are suitable for preparing gram scale amounts of highly purified HA oligosaccharides for use in biological applications.

8. Novel Methods for the Preparation and Characterization of Hyaluronan Oligosaccharides of Defined Length

This study describes methods for the purification of hyaluronan oligomers of defined size using size exclusion and anion-exchange chromatography following digestion of hyaluronan with testicular hyaluronidase. These preparations were characterized by a combination of electrospray ionization mass spectrometry, matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight analysis, and fluorophore assisted carbohydrate electrophoresis. Hyaluronan oligomers ranging from tetrasaccharides to 34-mers were separated. The 4- to 16-mers were shown to be homogeneous about length but did contain varying amounts of chondroitin sulphate. This contaminant could have been minimized if digestion had been performed with medical grade hyaluronan rather than the relatively impure starting material used here. The 18- to 34-mer preparations were mixtures of oligosaccharides of different lengths (e.g., the latter contained 87% 34-mer, 10% 32-mer, and 3% 30-mer) but were free of detectable chondroitin sulphate. In addition to oligomers with even numbers of sugar rings, novel 5- and 7-mers with terminal glucuronic acid residues were identified.

Digestion conditions

HA (100 mg) was dissolved at a concentration of 3.33 mg/mL (30 mL total) in digest buffer (0.15 M NaCl, 0.1 M Na-acetate, adjusted to pH 5.2 with glacial acetic acid) and incubated at 37 °C for 30 min. To this, **ovine testicular hyaluronidase** (0.22 mg; 11,000 U in 10 mL digest buffer) was added, and the solution incubated at 37 °C. The digest was assessed at various time points by running 100 µL of the reaction mixture (diluted to 3.5 mL with H_2O) on a 75 × 7.5 mm Waters PAK DEAE-5PW anion-exchange column, equilibrated in 5.0 mM ammonium hydrogen carbonate (buffer A) at a flow rate of 1 mL/min. The initial conditions were maintained for 10 min, and HA oligosaccharides were eluted with gradients of 0–50% and 50–95% of buffer B (500 mM ammonium hydrogen carbonate) in buffer A over 15 and 5 min, respectively. Final conditions were maintained for 5 min prior to re-equilibration into buffer A. The eluent was monitored at 210 nm. When the reaction reached the desired point, it was stopped by boiling for 5 min, dialyzed against 3 × 10 L H_2O for 6 h each at 4 °C, lyophilized, and stored at –20 °C.

9. Enzymatic production of specifically distributed hyaluronan oligosaccharides

High-molecular-mass hyaluronan (HA) was controllably depolymerized in pure aqueous solution with **recombinant leech hyaluronidase** (HAase). The HAase concentration per unit HA and hydrolysis time played important roles in molecular mass distribution. By modulating the concentrations of HAase and controlling the hydrolysis time, any molar-mass-defined HA oligomers could be efficiently and specifically produced on a large scale (40 g/L), such as HA oligosaccharides with weight-average molar mass of 4000, 10,000, and 30,000 Da and end hydrolysates containing only HA6 and HA4. High performance liquid chromatography-size exclusion chromatography, polyacrylamide gel electrophoresis, capillary zone electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry confirmed low polydispersity of the produced molar-mass-defined HA oligosaccharides. Therefore, large-scale production of defined HA oligosaccharides with narrow molecular mass distribution will significantly promote progress in related research and its potential applications.

10. High-yield novel leech hyaluronidase to expedite the preparation of specific hyaluronan oligomers

HAases have been the subject of intense research due to their considerable potential for use in biotechnological processes, particularly the pharmaceutical industry. The discovery of new HAases with novel properties is thus an important research area.

In this work, authors cloned **a novel leech HAase designated LHyal**. In silico analysis demonstrated that LHyal should be classified in glycosidase family 79 and is highly homologous to heparanases, indicating a common ancestor of these enzymes. Functional overexpression of LHyal confirmed its identity as a novel HAase. Here, by employing N-terminal engineering and fed-batch fermentation, a large amount of HAase was obtained, with a crude titre of 8.42 × 105 U/mL, a value much higher than that of commercial BTH (1,005 U/mg, 20–25% pure) extracted from bovine testicle. Compared with the acidic BTH14, the easily purified H6LHyal exhibits superior enzymatic properties (a near-neutral optimum pH of 6.5 and an optimum temperature of 45 °C, with excellent temperature resistance).

They successfully cloned the first leech hyaluronate 3-glycanohydrolase family (EC 3.2.1.26) gene and achieved its high-level heterologous overexpression. Compared with commercial BTH, this novel HAase has better substrate specificity and a vastly different hydrolysis mechanism. By controlling the incubation time, different HA oligosaccharides, particularly HA10, HA8, HA6 and HA4, can be selectively generated with high yields.

11. Construction of saturated odd- and even-numbered hyaluronan oligosaccharide building block library

Even-numbered o-HAs with two different reducing ends (HA2nAN and HA2nNA, n = 1, 2, 3…) can be prepared using **bovine testicular hyaluronidase** (BTH) and **leech hyaluronidase** (LHase) respectively, while preparation of odd-numbered o-HAs (HA2n+1AA and HA2n+1NN, n = 1, 2, 3…) is relatively difficult. A new enzymatic method was developed to prepare odd-numbered o-HAs in this study, taking advantage of differences in the degradation of glycosidic linkages by BTH and LHase. Through quantitative analysis of the yield of even-numbered o-HAs, a dynamic model for LHase hydrolysis was established, providing optimal preparation conditions for HA2nNA (n = 1–5). Then HA2nNA (n = 2–5) was further degraded by BTH to produce odd-numbered o-HAs. The above o-HAs were separated and purified by combining high-performance Q-Sepharose ion-exchange (Q HP) and size exclusion column chromatography steps.

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

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. The specific activity of crude hyaluronidase solution reached 2.3×106 U/mg. The optimum enzymatic activity was achieved at 37 ℃ and p H 5.5.

13. Preparation and extensive characterization of hyaluronan with narrow molecular weight distribution

The physicochemical properties and biological functions of hyaluronan (HA) are closely related to its molecular weight (MW) and molecular weight distribution (MWD). Therefore, it is crucially important to provide a reliable characterization of these parameters for proper use of HA and its degradation products in both chemical and clinical fields. In this study, authors present a novel method for the preparation of HA fragments of defined size with narrow molecular weight distribution.

The HA fractionation was performed using an anion-exchange chromatography and is applicable either after enzymatic or chemical hydrolysis of polymeric HA. Isolated fractions with a molecular weight ranging from 3000–420,000 g/mol were analyzed by size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS). Hundred-milligram

scale HA fragments were obtained from 5 g hyaluronan starting material. Independently on weight-average molecular weight (Mw), the polydispersity index (PDI) of the HA fractions was less than 1.23. The fractionation methodology can be easily up-scaled and is applicable on any negatively charged polymers.

14. A novel hyaluronidase produced by *Bacillus sp. A50*

The hyaluronidase (**HAase-B**) was isolated and purified from the bacterial culture, with a specific activity of 1.02×10^6 U/mg protein and a yield of 25.38%. The **optimal temperature and pH of HAase-B were 44 °C and pH 6.5**, respectively. It was stable at pH 5–6 and at a temperature lower than 45 °C. The enzymatic activity could be enhanced by Ca^{2+}, Mg^{2+}, or Ni^{2+}, and inhibited by Zn^{2+}, Cu^{2+}, EDTA, ethylene glycol tetra acetic acid (EGTA), deferoxamine mesylate salt (DFO), triton X-100, Tween 80, or SDS at different levels. Kinetic measurements of HAase-B towards HA gave a Michaelis constant (Km) of 0.02 mg/mL, and a maximum velocity (Vmax) of 0.27 A232/min. HAase-B also showed activity towards chondroitin sulphate A (CSA) with the kinetic parameters, Km and Vmax, 12.30 mg/mL and 0.20 A232/min respectively. Meanwhile, according to the sequences of genomic DNA and HAase-B's part peptides, a 3,324-bp gene encoding HAase-B was obtained.

15. Characterization of Hyaluronan-Degrading Enzymes from Yeasts

The study elucidated that hyaluronate 4-glycanohydrolase and hyaluronan (HA) lyase can be produced by yeasts. Six yeasts producing HAases were found through express screening of activities. The extracellular **HAases** from two of the yeast isolates, ***Pseudozyma aphidis*** and ***Cryptococcus laurentii***, were characterized among them. *P. aphidis* HAase hydrolyzed β-1,4 glycosidic bonds of HA, yielding even-numbered oligosaccharides with N-acetyl-D-glucosamine at the reducing end. *C. laurentii* produced hyaluronan lyase, which cleaved β-1,4 glycosidic bonds of HA in β-elimination reaction, and the products of HA degradation were different-sized even-numbered oligosaccharides. The shortest detected HA oligomer was dimer. The enzymes' pH and temperature optima were pH 3.0 and 37–45 °C (*P. aphidis*) and pH 6.0 and 37 °C (*C. laurentii*), respectively. Both HAases showed good thermostability.

16. Hyaluronic acid of tailored molecular weight by enzymatic and acid depolymerization.

While a number of enzymatic, chemical and physical methods exist for HA depolymerization, limited information is currently available for accurate planning of experiments. In the present work, they propose a pseudo-mechanistic model to describe depolymerization kinetics of HA with hyaluronidase, chondroitinase ABC and phosphoric acid. Data to feed the model was provided by monitoring molecular weight reduction by gel permeation chromatography with light scattering detection over 24 h. Five enzyme to substrate ratios and three temperatures were used for enzymatic and chemical reactions respectively, allowing for selection of operational parameters in a range of conditions. The model adequately reproduces the resulting data providing flexibility in the planning of the reactions to obtain HA of the desired molecular weight.

Hyaluronic acid (HA) was produced by microbial fermentation from ***Streptococcus equi* subsp. zooepidemicus ATCC 35246**. Then, HA was isolated and purified from bacterial post-incubates by a combination of chemical and ultrafiltration treatments to obtain HA of purity greater than 98% and 1.2 MDa of molecular weight. This final HA was depolymerized with **chondroitinase ABC** (CASE) from *Proteus vulgaris* (EC 4.2.2.4., 0.86 U/mg, Prod. No. C2905, Sigma-Aldrich), **hyaluronidase (HASE**) from bovine testes type I-S (EC 3.2.1.35., 587 U/mg, Prod. No. H3506, Sigma-Aldrich), and phosphoric acid (85%, Prod. No. 20624.295, VWR Chemicals). HA was dissolved at 1 g/L, in 50 mM TRISHCl / 150 mM sodium acetate at pH 8 for reaction with CASE; in 5 mM NaH_2PO_4 / 150 mM NaCl at pH 4 for HASE depolymerization; and in ultrapure water for hydrolysis with H_3PO_4. Enzymatic reactions were carried out in triplicate at 37 °C under magnetic stirring by adding the appropriate amounts of either enzyme to tubes containing 10 mL of HA solutions to reach enzyme to substrate ratios of 0.01, 0.025, 0.05, 0.075 and 0.01 units of CASE and 0.5, 1.0, 2.0, 7.0 and 12.0 units of HASE per mg of HA. Acid hydrolysis was performed in duplicate after addition of 5 mg H_3PO_4 per mg HA (pH 1.71) to 10 mL of 1 g/L HA solutions at 40 °C, 60 °C and 75 °C.

17. Characterisation of separated end hyaluronan oligosaccharides from leech hyaluronidase and evaluation of angiogenesis.

Hyaluronan oligosaccharides (o-HAs), especially saturated o-HAs, have attracted intensive attention due to their potential applications in medical treatments. In this study, the hydrolysis process of **leech hyaluronidase (LHase)** towards the hyaluronan was investigated by HPLC and HPLC/ESI-MS. The proportions of hyaluronan tetrasaccharide (HA4) with hexasaccharide (HA6), end products, were illustrated to have a relationship with the amount of LHase. Higher yield of HA4 was achieved with higher activity of LHase. After optimisation of the packing resin and operation parameters (balanced pH, elution concentration, elution volume and elution flow rate), the highly pure HA4 and HA6 were efficiently separated and prepared by combining ion exchange Q-Sepharose Fast Flow and size exclusion column chromatography. Compared with o-HAs (average Mr of 4000 Da), HA4 and HA6 were demonstrated to show higher activity for promoting angiogenesis, which was similar with the corresponding HA4 and HA6 produced by bovine testicular hyaluronidase. The pure HA4 and HA6 that prepared from LHase will attract intensive studies and be used in potential applications in near future.

LHase kept high activity when incubated at 38 °C for several hours. As a result, one gram HA was hydrolysed by a series of doses of LHase (4000–32,000 U/mL) in 100 mL distilled water with different incubation time at 38 °C, respectively. 1 mL sample was taken out at 0.5, 1, 2, 4, 8, 12, 16, 24, 32 and 40 h, and the enzymatic treatment for HA was stopped by boiling the samples for 20 min.

The samples were centrifuged by ultracentrifugal filters with a cut off molecular weight of 3000 Da (from Millipore, Billerica, MA, USA) to separate o-HAs with those molecular weight larger than 3000 Da, and the ultracentrifugation filtrate were collected. o-HAs of different incubation time were then lyophilised and dissolved in 1 mL of distilled water to give the final products.

4. Annex

SEQ ID NO: 1 (DOCUMENT 2. US2015175991 AA)

NESTLLLNTSFEETEAPKSGWDQLGAPKWGVVWRPTGSPIVTITKEASRTGEYGLKIAAAQSARAAVSQDVPVQGGQTYQL
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SEQ ID NO: 2 (DOCUMENT 4. CN112501088 A)

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LVEAKSPGTAVISVETADGTVKSESQVTVTPAALDEYDLLRHKWENQMTSLDYYDASNERMKQMVASQTKSAQSLWRT
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SPHSIR

SEQ ID NO: 3 (DOCUMENT 7. CN110331178 A)

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SEQ ID NO: 4 (DOCUMENT 10. US2015031085 AA)

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KK

SEQ ID NO: 5 (DOCUMENT 10. US2015031085 AA)

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K