Purification and Characterization of Hyaluronate Lyases Produced by Two Types of Bacteria

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Abstract Hyaluronate lyases were obtained from two types of naturally isolated bacterial strains *Paenibacillus yunnanensis* and *Paennarthrobacter nicotinovorans*. PyHL (form *P. yunnanensis*) in the culture supernatant of the bacteria was purified by two steps of column chromatography. The enzyme showed the molecular mass of 74 kDa by SDS-PAGE and the maximal activity at pH 5.0, 35℃. PyHL maximally degraded hyaluronate by an endo-type manner, and showed low degradation activity toward chondroitin sulfates. Dermatan sulfate was not the substrate. PnHL (from *P. nicotinovorans*) in the culture supernatant of the bacteria was purified by two steps of column chromatography. The enzyme showed the molecular mass of 70 kDa by SDS-PAGE and the maximal activity at pH 6.0, 30 ℃. Genomic analysis of *P. nicotinovorans* on the bases of the internal amino acid sequences of PnHL.

Key words Glycosaminoglycan; Hyaluronan; Hyaluronate lyase; *Paenibacillus yunnanensis*; *Paennarthrobacter nicotinovorans*

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Hyaluronate (HA) is an anionic linear polysaccharide composed of repeating disaccharide units of [(1→3) β-D-N-acetylglucosamine-(GlcNAc)-(1→4) β-D-glucuronic acid(GlcUA)], and classified as a glycosaminoglycan without sulfate residues. HA is abundantly distributed in nearly all vertebrate tissues, especially extracellular matrix (ECM). HA is also produced by some bacteria of the genus *Streptococcus*[1-2].

HA is degraded by hyaluronidases, which were classified into three main families on the basis of their catalytic mechanisms of action[3]. Hyaluronidases (EC 3.2.1.35) degrade HA by hydrolysis of the B-1,4-glycosidic bond furnishing tetrasaccharide molecule as the main product. These enzymes were extracted from vertebrate tissues and venom. Hyaluronidases (EC 3.2.1.36) are mainly extracted from hookworms and the salivary glands of leeches. These enzymes degrade HA by hydrolysis of the B-1,3-glycosidic bond, thus, yielding sugar fragments having glucuronic acid at the reducing end and generate tetra- and hexasaccharide end products. Hyaluronidases (EC 4.2.2.1), also known as hyaluronate lyases (HL), were isolated from various microorgansims, including e.g. strains of *Clostridium*, *Streptococcus*, *Streptomyces*, *Arthrobacter*, and *Bacillus*[4-9]. They degrade HA by a B-elimination reaction to yield unsaturated hyalurono-oligosaccharides as the exhaustive degradation products.

Hyaluronidases are widely used in clinical treatment, medical science, and biochemical research. Hyaluronidases are considered as a "spreading factor", which increases membrane permeability for rendering tissues more permeable to injected fluids by degrading HA in the ECM. Therefore, the enzyme is approved as an adjuvant for increasing analgesic efficacy[10]. In the clinic, hyaluronidases could be applied to vitreous opacity, glaucoma and control vascular complications after HA filler injection in aesthetic dermatology[11-12]. And in biochemical researches, hyaluronidase can be used as tools for the identification of HA[13] and producing HA oligosaccharides[14].

The current commercial hyaluronidases are mainly prepared by extracting bovine testicular tissue. However, the purity and high price of these enzymes limit their usage in pharmaceutical and biochemical engineering[15]. Therefore, more attentions are paid to the hyaluronidases (HL) from bacteria.

In this study, *Paenibacillus yunnanensis* strain16-6 and *Paennarthrobacter nicotinovorans* strain 19-1 were isolated from the Lake in Aomori, Japan, and were chosen for those degradation activity toward HA. The hyaluronate lyases produced by the strain (PyHL, PnHL) were purified, characterized. The results showed that PyHL and PnHL could be effective tools for analyses of HA and preparation of HA oligosaccharides, which have the potential to be used in drugs or biochemical research.

Material and Methods

Materials

Bacterial strains (i) *P. yunnanensis* strain 16-6 was obtained from water of Ketobanoike, a pond in Lakes Juniko (Aomori pref., Japan) by an enrichment culture with HA as the substrate. Subculture was repeated until a single colony was obtained. Bacterial identification on the basis of 16S rRNA analysis was performed by Macrogen Corp. (Japan).

(ii) *P. nicotinovorans* strain 19-1 was isolated from water of Lake Towada, (Aomori Pref., Japan) by an enrichment culture with PG as the substrate. The rest of the experimental methods

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were the same as those of *P. yunnanensis* strain 16-6.

Reagents Low-molecular weight HA was purchased from Healthy Co. (Japan), and used for bacterial culture. HA from rooster comb (Fujifilm Wako Pure Chemical Co., Japan) was used. Toyopearl HW55F and DEAE Toyopearl 650M were purchased from Tosoh Bioscience Co. (Japan). Proteoglycan (PG) was generously provided by Glycosmo Co. (Japan). Other reagents were commercially supplied by Fujifilm Wako Pure Chemical Co. (Japan).

Microbial cultivation and purification of PyHL and PnHL

(i) *P. yunnanensis* strain 16-6 was grown in the liquid medium (0.1% HA, 0.67% Yeast Nitrogen Base (w/o) Amino Acid, 0.02% Yeast Extract) with an initial pH 7.0. After 48 h of culture at 27℃ with shaking in a 1 L flask, bacterial cells were removed by a centrifugation of the broth for 20 min at 8 000 rpm at 4 ℃. Following steps were carried at 4 ℃. Proteins in the supernatant were precipitated by ammonium sulfate at the concentration of 70% saturation, then centrifuged for 20 min at 8 000 rpm. The pellets were dissolved in 10 mM acetate buffer (pH 6.0) containing 0.1 M NaCl, then applied to a column of Toyopearl HW55F column that was wan in the same buffer. The enzyme was pooled and concentrated using an ultrafiltration tube (cut by 30kDa, Macrosep® Advance Centrifugal Devices 30 K, Pall Co. USA), and then loaded onto a DEAE Toyopearl 650 M column that was pre-equilibrated in 10 mM acetate buffer (pH 6.0). The elution was performed with a liner gradient of NaCl (0.0 – 0.3 M) in the same buffer. PyHL was concentrated using an ultrafiltration tube (cut by 30 kDa, Nanosep® 30 K Omega, Pall Co. USA) and kept frozen until use. Protein concentration was determined by a method developed by Bradford using bovine serum albumins as the standard[16]. SDS-PAGE was performed according to the method described by Laemmli using 10% polyacrylamide gels[17].

(ii) *P. nicotinovorans* strain 19-1was grown in the liquid medium (0.1% PG, 0.67% Yeast Nitrogen Base (w/o) Amino Acid, 0.02% Yeast Extract) with an initial pH 7.0. After 48 h of culture at 27 ℃ with shaking in a 1 L flask, bacterial cells were removed by a centrifugation of the broth for 20 min at 8 000 rpm at 4 ℃. Following steps were carried out at 4 ℃. Proteins in the supernatant were precipitated by ammonium sulfate at the concentration of 75% saturation then centrifuged for 20 min at 8 000 rpm. And the purification method was basically the same as PyHL above.

Protein concentration and SDS-PAGE, Native-PAGE were performed according to the method as PyHL.

Determination of hyaluronate lyases activity (PyHL, PnHL)

Reaction mixtures containing 0.01% (w/v) HA and the enzyme in total 1.0 ml of 50 mM sodium acetate buffer (pH 5.0), were incubated at 30 ℃ for a period of time. The reactions were stopped by adding 0.5 mL of 1 M NaOH, and then UV absorption of the solutions were measured at 232 nm. One unit (U) of HL activity was defined as the amount of the protein required of the eliminative cleavage of substrate, yielding UV-absorbing materials corresponding to 1 μmol of unsaturated double bond per minute. A millimolar absorption confficient of 5.5 was used in all calculations[18-19].

Effects of pH and temperature on the activity and stability of PyHL and PnHL

(i) The optimum pH of PyHL was determined by measuring the activity of HL with 0.02 M buffer including sodium acetate-HCl (pH 1.0 – 4.0), sodium acetate-acetate (pH 4.0 – 6.0), sodium phosphate (pH 6.0 – 8.0), Tris-HCl (pH 8.0 – 9.0), disodium carbonate (pH 9.0 – 10.0), and sodium tetraborate-NaOH (pH 10.0 – 12.0). Similarly, a range of 10 – 70 ℃ was used to determine the optimum temperature for PyHL. Thermal stability and pH stability were measured by incubating the enzyme at different temperatures (10 – 70 ℃) and buffers (pH 1.0 – 12.0), respectively. The residual activity was measured under standard conditions described above.

(ii) PG was used as the reaction substrate and the experimental method was as same as PyHL.

Substrate specificity

(i) PyHL as 50 μl enzymatic solution was added to a reaction mixture containing 1.95 ml substrate solution (CSA, CSB, CSC, HA, PG, or GAG, 1mg/ml) in the 0.05 M acetate buffer (pH 5.0). After incubation at 30 ℃ for 60 min, the reactions were stopped by adding 0.5 ml of 1 M NaOH. Then the UV absorption of the resultant solutions was measured at 232 nm.

(ii) The experimental method was as same as PyHL.

HPLC of PyHL

A reaction mixture composed of 0.01% HA, 0.05 M acetate buffer (pH 5.0), and the enzyme was incubated at 30 ℃ from 30 min to 12 h. A change in the size of HA was monitored by gel permeation HPLC with TOSOH TSK-gel G5000PWXL column (Tosoh Co., Japan) that was ran in 0.2 M NaCl solution (0.5 ml/min). Effluent was monitored by Refractive Index monitor (Model Chromaster 5450, Shimazu Co., Japan).

Protein analysis of PnHL

The protein analysis of PnHL was recovered from the polyacrylamide gel after performing SDS-PAGE, and subjected to amino acid sequence analysis by LC/MSMS. Internal amino acid sequences of the tryptic peptides were identified by LC-MS/MS (Triple TOF 6600, AB Sciex, Japan) with ProteinPilot Software (AB Sciex) for data processing. The amino acid sequence of PnHL was determined by informatics works with the genomic information of *P. nicotinovorans* that was used as a database in the MS/MS spectra search. By using the Prosite of ExPASy (http://prosite.expasy.org/) the protein function was predicted from the amino acid sequence.

Results and Analysis

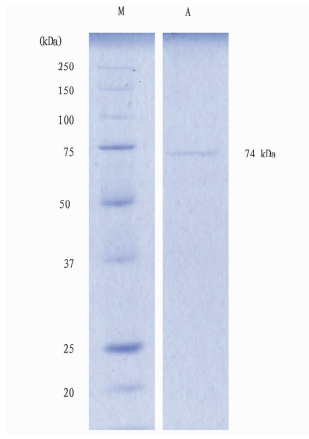
Molecular and enzymatic properities of hyaluronate lyases (PyHL, PnHL)

(i) The enzyme PyHL was purified from the culture broth *P. yunnanensis* by two steps of column chromatography (Table 1).

PyHL appeared as a single band on SDS-PAGE and the molecular weight was estimated approximately 74 kDa (Fig. 1). PyHL showed the maximal activity at pH 5.0 in an acetate buffer and almost no activity was shown below pH 3.0 and above pH 9.0 (Fig. 2a, c). We observed an inhibitory effect by a phosphate buffer that reduced the activity by a half of the acetate at pH 6.0. Over 70% of the initial activity was kept after pretreatment at pH between pH 5 and 8 with the maximal at pH 6.0. The activity of PyHL at pH 5.0 was maximal at 35℃ and was stable after a preheating below 45℃ (Fig. 2b, d).

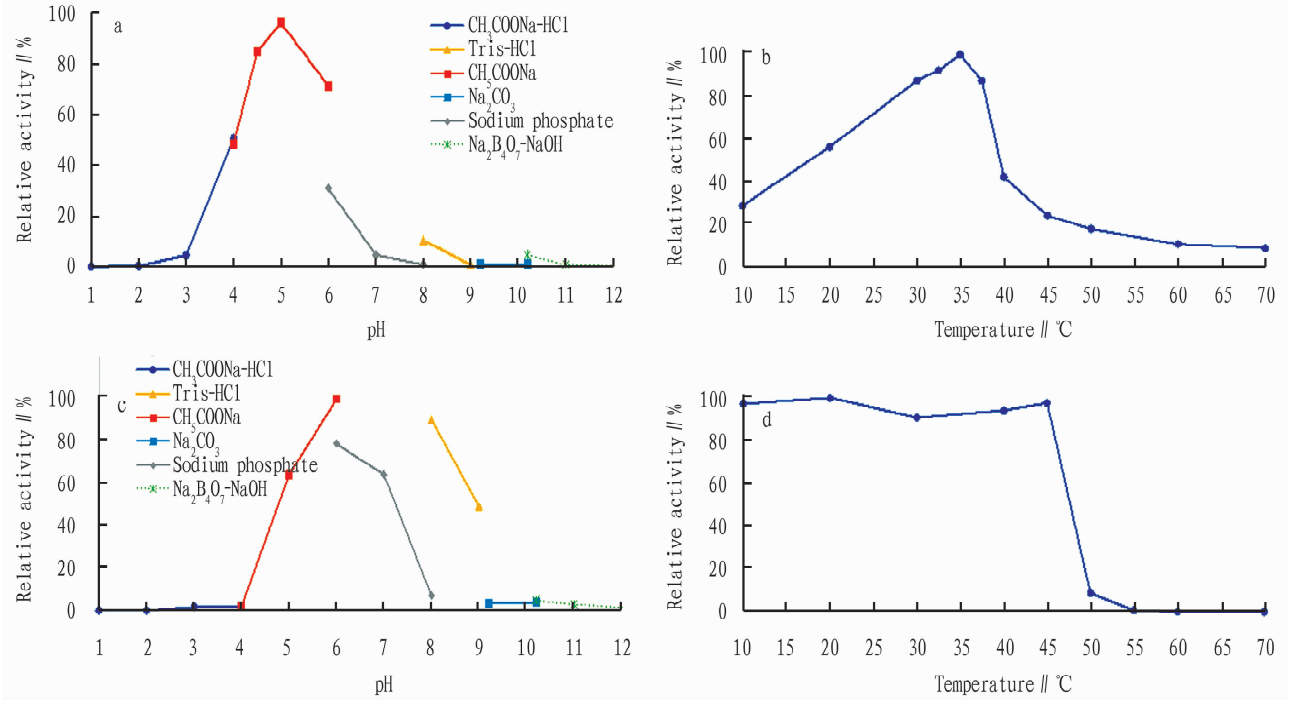
Table 1 Summary of PyHL purification

Steps	Total protein mg	Total activity U	Specific activity U/mg	Yield %	Purification fold
Crude	5.6	1 805.8	323.8	100.0	1.0
Gel permeation	2.1	674.5	326.1	37.4	1.0
DEAE Toyopearl 650M	1.0	469.1	473.9	26.0	1.5



Lane M: Molecular mass markers; lane A: purified PyHL.

Fig. 1 SDS-PAGE of hyaluronate lyase from *Peanibacillus yunnanensis*.



a. Effects of pH on the activity of PyHL: The activity was measured at 30℃ in a broad pH range buffer as described previously. c. pH stability of PyHL: After incubation at various pH buffer solutions for 30 min at 30℃, the residual activity was assayed in acetate buffer (0.5 M, pH 5.0) at 30℃. b. Effects of temperature on the activity of PyHL: The activity of PyHL was measured at different temperatures. d. Effects of temperature on the stability of PyHL.

Fig. 2 Effects of pH and temperature on the activity and stability of PyHL

(ii) The enzyme PyHL was purified from the culture broth *P. yunnanensis* by two steps of column chromatography (Table 2). PyHL appeared as a single band on SDS-PAGE and the molecular weight was estimated approximately 70 kDa (Fig. 3). PnHL showed the maximal activity at pH 5.0 in an acetate buffer and almost no activity was shown below pH 4.0 and above pH 9.0 (Fig. 4a, c). The activity of PyHL at pH 5.0 was maximal at 35 ℃ and was stable after a preheating below 40℃ (Fig. 4b, d).

Substrate specificity of PyHL and PnHL

(i) PyHL exhibited the highest activity toward HA among the GAG substrates (Table 3). The digestion ratios of CSA, CSC were respectively 7% and 9% of HA and the degradation of dermatan sulfate was negligible.

Bacterial HL could be divided into three types of enzymes depending on their substrate specificities. HL from *Streptomyces hyalurolyticus* are specific to HA, while *Streptococcus dysgalactiae* HL show a little activity toward CSA and CSC[21-22]. Arthrobacter HL degrades CS with nearly one third of the activity toward HA[23]. In this context, PyHL was supposed to have a similar specificity with *Peptostreptococcus* HA lyase.

(ii) PnHL exhibited the highest activity toward HA among the GAG subatrates (Fig. 5). Then PnHL had degradation activity for CSC and CSA, but had no activity for DS.

Table 2 Summary of PnHL purification

Steps	Total protein mg	Total activity U	Specific activity U/mg	Yield %	Purification fold
Crude	2.84	406.77	143.48	100.00	1.00
Toyopearl HW 55 Fine	1.83	179.09	97.72	44.03	0.68
DEAE Toyopearl 650M	1.16	144.00	123.68	35.40	0.86

Table 3 Substrate specificity of PyHL

Substrate	Relative activity//%
HA	100
CSA	7.4
CSC	9.1
DS	0
PG	22.4
GAG	10.6
Desulfurized GAG	31.9

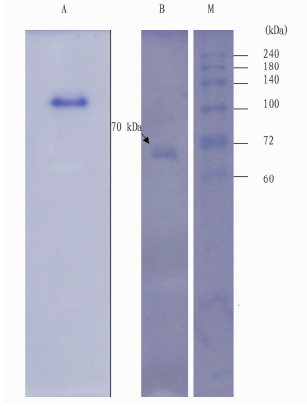
Determination of degradation products

The reaction mixtures were analyzed by HPLC, monitored at 232 nm which was specific for unsaturated bands. The time sequence changes in the reaction mixtures when the HA oligosaccharide was used as the substrate are showed in Fig. 6 as an example. Decasaccharides and tetrasaccharides were detected at 232 nm, but no disaccharides were detected by HPLC.

Studies have showed that HA oligosaccharides have important application prospects in the field of medicine[24], such as applications in promotion of angiogenesis[25], immunomodulation, and anti-tumor[26].

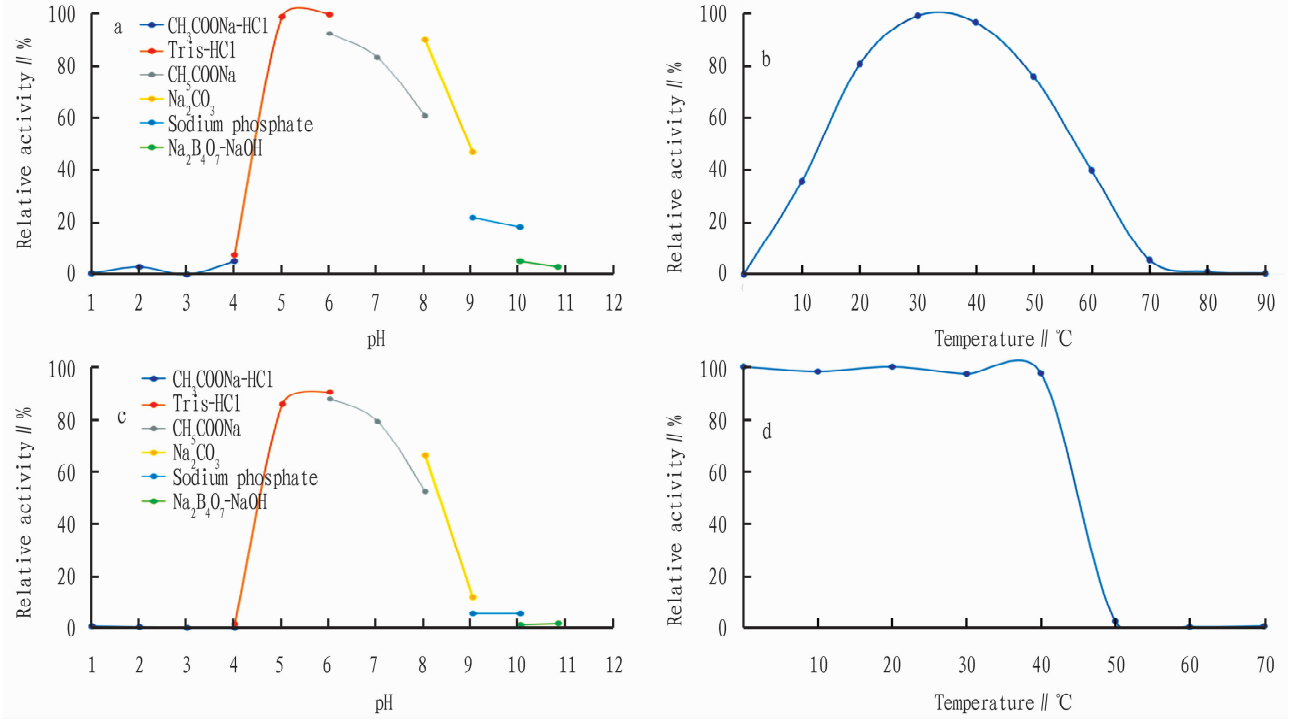
Protein analysis of PnHL

PnHL protein was recovered from polyacrylamide gels after SDS-PAGE and amino acid sequence analysis was performed by LC-MS/MS. The identified amino acids were sequenced according to the genomic data of strain 19-1, and the resulting proteins were searched for homology by BLAST (Fig. 7). The results showed that PnHL is highly homologous to *P. nicotinovorans*[27] polysaccharide lyase 8 family protein (hyaluronate lyase).



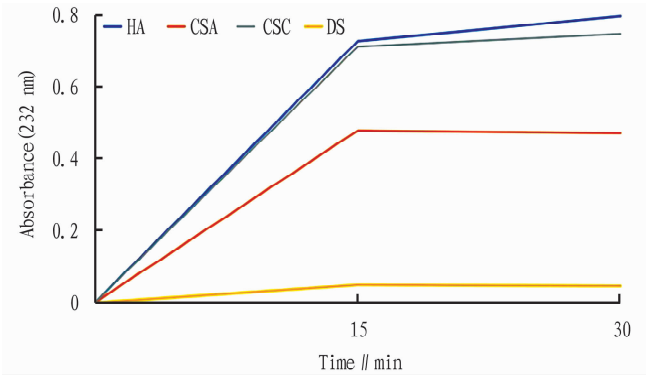
A: Native-PAGE of purified PnHL; B: SDS-PAGE of purified PnHL; M: molecular mass markers.

Fig. 3 Native-PAG and SDS-PAGE of purified PnHL



a. Effects of pH on the activity of PnHL: The activity was measured at 30℃ in a broad pH range buffer as described previously. b. Effects of temperature on the activity of PnHL: The activity of PnHL was measured at different temperatures. c. pH stability of PnHL: After incubation at various pH buffer solutions for 30 min at 30℃, the residual activity was assayed in acetate buffer (0.5 M, pH 5.0) at 30℃. d. Effects of temperature on the stability of PnHL.

Fig. 4 Effects of pH and temperature on the activity and stability of PnHL



Reaction mixtures containing 0.01% (*w/v*) of HA, CSA, CSC, and DS were treated with PnHL at pH 5.0, 30 ℃ for a period of time. UV absorption of the reaction mixtures was measured at 232 nm.

Fig. 5 Substrate specificity of PnHL

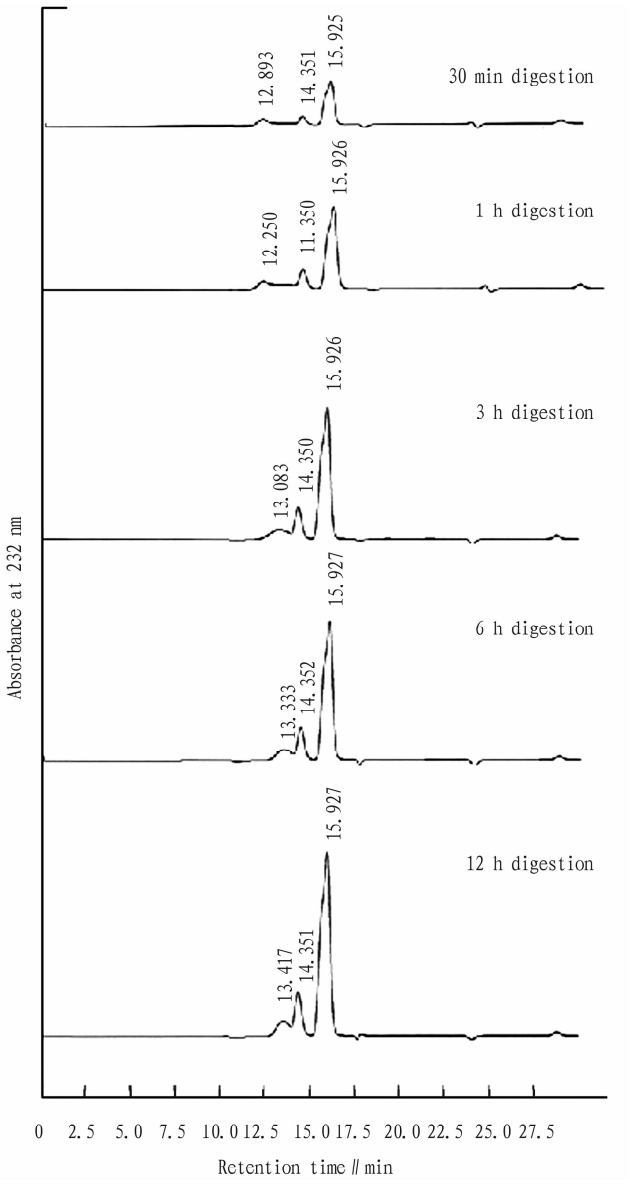
Conclusions and Discussion

In this study, two types of extracellular hyaluronate lyase were successfully purified to homogeneity from fermentation broth of the bacteria *P. yunnanensis* and *P. nicotinovorans*. These enzymes had the approximate molecular weight of 74 and 70 kDa by SDS-PAGE, which is similar to the known HL, such as 73.7 kDa (*Arthrobacter globiformis* A152)[8], 77 kDa (*Streptomyces hyalurolyticus*)[20], but different with known HL, such as 120 kDa (*Bacillus niacini* strain JAM F8)[9], 50 kDa (group A type 4 *Streptococcus*)[29], 107 kDa (*Streptococcus pneumoniae*)[28], and 114 kDa (*Clostridium perfringens*)[4]. Characteristics of these enzymes were also studied. The hyaluronate lyase also displayed pretty good levels of activity and stability.

Hyaluronate lyases from various species showed different specificities towards polysaccharide substrates[30]. Both PyHL and PnHL showed highest substrate specificity towards HA, which was different from the other conventional hyaluronidases. Generally, the hyaluronate lyase (EC 4.2.2.1) can typically degrades HA and is relatively less active against CSs. Although, the HCLaseM (M, sp. H14) could degrade HA and various types of CSs and showed almost the same enzyme activity towards CS-A, CS-B, CS-C and HA (98, 97, 96, and 100), the PyHL and PnHL (this study) showed lowest activity towards CSs, which showed higher level of substrate specifity on HA. PyHL and PnHL may have the potential to be an effective tool for preparation of functional oligosaccharides and investigation function relationship of HA.

Amino acid sequence analysis was performed on PnHL by LC-MS/MS and the results were checked against the whole genome analysis data of *P. nicotinovorans* strain 19-1. The results showed that 377 amino acids were identified with 95% confidence, accounting for 82.9% of the protein sequence of PnHL. The protein identified by homology analysis showed high homology to *P. nicotinovorans* polysaccharide lyase family 8 protein (hyaluronate lyase, WP_189076633.1). Therefore, in this study, we have chosen to refer to it as hyaluronate lyase.

Hyaluronidase as a glycosaminoglycan lyase, has recently attracted attention for its use in the pharmaceutical industry and clinical medicine for the treatment and improvement of joint conditions, and will likely play an important role in biotechnological



The reaction mixture was incubated in 0.5 M Acetate buffer (pH 5) and 0.1% HA at 30 ℃ from 30 min to 12 h with enzyme. The reaction products were analyzed by HPLC on a hydroxylated polymethacrylate column (7.8 mm × 300 mm). The column was eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min. Oligosaccharides were detected by UV absorbance at 232 nm.

Fig. 6 HPLC analysis of degradation products of HA by PyHL

LSLQFPRRTLLQGAGALSLAAVVSSMFAQNAWAEAEPGAAEFAALRNRWVDQITGRN
VIQAGDPDFAKAITSLNNKAADSLAKLNTSSGRTSVFTDLSLAKDAEMVTTYTRLSQLA
TAWATPTAAVFGDAAVLAAIKAGLADANTLCYNDRKEEVGNWWSWEIGVPRALADA
MVLLHAELSAAERTAYCAAIDHFVPDPWLQFPPKRGKITSVGANRVDLCQGIIRSLAG
EDPAKLNHAVAGLSQVWQYVTSGDGIFRDGSFIQHSTTPYTGSYGVVLLTGLSKLFSLL
GGTAFEVSDPTRSIFFDAVEGSFAPVMINGAMADAVRGRSISREANTGYDLGASAIEAIL
LLARAMDPATAARWRGLCAGWISRNTYRPILNSASVPRTALVKELQATGVAPVAEATG
HKLFPAMDRTMHRGPGWALSLSMSSNRIAWYECGNGENNRGYHTGSGMTYFYTSDL
GQYDDAFWATANYNRLPGTTVDTTPLPDKVEGEWGAAVPANEWSGATTLGEVAAV
GQHLVGPGRTGLTARKSWFVSGDVTVCLGADISTASGARVETIVDHRNLHQGSNTLT
TAAGTIAGTAGTVEVLGDGRWVHLEGFGGYAMLDDSPLHVLRETRSGSWSGVNINGS
ATVQQRNFATIYVDHGVGPVAGSYAYMVAPGASVDLTRKLLQGNKYRVIRNDTTAQS
VEFKTAKTTAATFWKPGMAGDLGASGPACVVFSRHGNELSLAVSEPTQKAAGLTLTL
PEGTWSSVLEGAGTLGTDADGRSTLTLDTTGLSGQTKLIKLRR

Amino acids identified by LC-MS/MS analysis are shown in green. Those with slightly lower reliability are shown in yellow and red.

Fig. 7 Amino acid sequence analysis of PnHL

research[31]. Since aggrecan, which is a PG abundant in joints, is enriched in CSA and CSC, PyHL and PnHL, which showed high degradation activity against them, are suitable for enzymatic therapy to treat and improve herniation in joints. In addition, the biological activity of low molecular weight hyaluronan oligosaccharides is superior to that of natural polymeric hyaluronan, hyaluronidases will be a major tool for the preparation of hyaluronan oligosaccharides. The discovery of new glycosaminoglycan lyases with various properties from nature and a better understanding of their functions and properties would be important not only for basic research but also from an applied perspective.

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