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Purification and Characterization of High Viscous Hyaluronic Acid Complex from *Klebsiella* sp. L-10 NTG 50

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Klebsiella sp. L-10 의 NTG 50 변이주로부터 생산된 고점성 히알우론산 복합체의 정제 및 특성

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Abstract

High viscous hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant was purified by two-phase extraction system using PEG-K_2HPO_4 and its physicochemical properties were investigated. Viscosity of the purified hyaluronic acid complex was decreased as temperature and salts concentration were increased and also showed low viscosity at below pH 5.0 and above pH 11.0. Hardness, cohesiveness and adhesiveness of the purified hyaluronic acid complex were 1.20kg, 1.91 and 0.62, respectively. Water holding capacity was 6.9ml per gram of the purified hyaluronic acid complex powder.

Key words : purification, high viscous hyaluronic acid complex, *Klebsiella* sp. L-10 NTG 50, physicochemical properties.

Introduction

Hyaluronic acid is composed of repeating units of glucuronic acid and *N*-acetylglucosamine, bound by alternating β-1,4 bonds. It is naturally distributed in connective tissue and skin of mammalians, eye, articular sac, cockscomb, placenta wall of main antesy, and capsule of *Streptococcus* hemolytics A and C group[1].

Because hyaluronic acid lubricates joints and absorbs the external shocks and especially it retains water in skin, it is widely used as drug delivery, orthopedics, ophthalmic surgery, cardiovascular aids, wound healing and cosmetics[1,2].

Although purification or extraction, utilization of hyaluronic acid from connective tissue or cockscomb and biosynthesis, cloning and sequencing of hyaluronic acid from *Streptococcus* sp. A and C group have been extensively studied[3~23], only a few studies have been reported on the purification and characterization of hyaluronic acid from other microorganisms except *Streptococcus zooepidemicus*[8], *Streptococcus equi*[6] and *Streptococcus pyogenes*[4].

We have previously reported on isolation and identification of high viscous hyaluronic acid complex-producing *Klebsiella* sp. L-10, optimization of production condition[21] and chemical mutagenesis of *Klebsiella* sp. L-10[23]. Here we describe on purification and characterization of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant.

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Materials and Methods

1. Strain and cultivation

The strain used in this study was *Klebsiella* sp. L-10 NTG 50 mutant, which *Klebsiella* sp. L-10 was treated with *N*-methyl-*N'*-nitro-*N''*-nitrosoguanidine(MNNG) and increased productivity of the hyaluronic acid complex to 2.5 folds[23].

The cultivation was carried out in YTD medium containing 0.1% yeast extract, 3.0% Bacto-trypton, 3.0% dextrose, each 30mM of K_2HPO_4 and KH_2PO_4(pH 6.0~6.5) at 37℃ for 24 hrs.

2. Purification of hyaluronic acid complex

Hyaluronic acid complex from *Klebsiella* sp. L-10 NTG-50 was purified by using two-phase extraction system of Jung et al.[9] as Fig. 1.

3. Measurement of viscosity and rheological properties

1) Viscosity

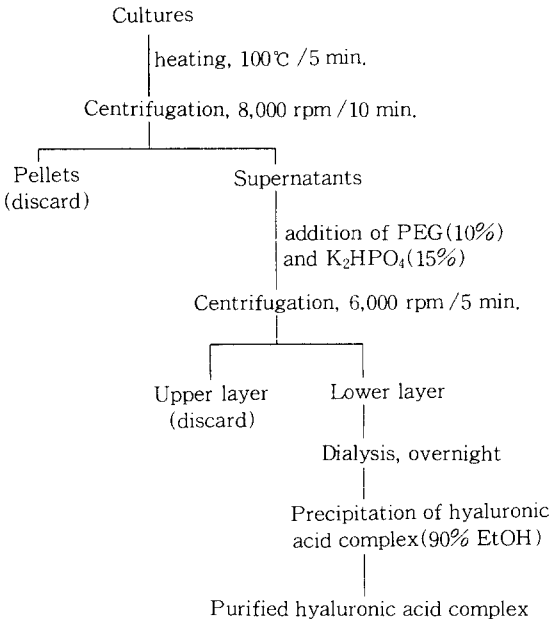

Fig. 1. Purification procedure of hyaluronic acid complex by using two phase extraction system.

Viscosity of 1% purified hyaluronic acid complex solution was determinated by Stomer viscometer (Arthur-H-Thormas Co., USA) at 10℃ to 90℃ and pH 1.0 to 13.0.

Effect of salts on viscosity was investigated after each 0.05% to 1.0% of KCl, NaCl, $CaCl_2$, $MgCl_2$, $FeCl_3$ were added in 3% hyaluronic acid complex solution.

2) Hardness, adhesiveness and cohesiveness

Each 30ml and 50ml of distilled water was added in 100g of hyaluronic acid complex powder and formed 0.8mm cubic. Texture profile analysis(TPA) curve of the cubic was obtained from 2 times compression repeating test by using Universal Test Machine(Instron Model 1000, Instron Engineering Co., Cauton, USA) and calculated hardness, adhesiveness and cohesiveness[24]. TPA curve and measurement conditions for Universal Test Machine were shown in Fig. 2 and Table 1, respectively(Fig. 2).

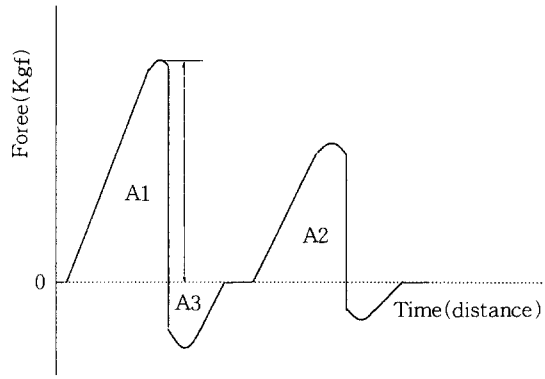

Fig. 2. Textural profile analysis curve. Hardness : height of A1, Adhesiveness : area of A3, Cohesiveness : area of A2 /area of A1.

Table 1. Measurement conditions for Universal testing machine

Sample height	25 mm
Sample diameter	0.8 mm
Clearance	4.0 mm
Chart speed	100 mm /min
Load cell	50 kg
Cross head speed	100 mm /min
Plunger diameter	12 mm

3) Water holding capacity

According to the Lin's method[25] distilled water was added in 1g of hyaluronic acid complex powder and shaked for 30 min. after centrifuged, free water determined and calculated absorption content of water.

Result and Discussion

1. Purification of hyaluronic acid complex

Hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 was purified by two phase extraction system using 10% PEG and 15% K_2HPO_4 and its recovery was 96%.

The purified hyaluronic acid complex was dried under vaccum to white powder. Glucuronic acid and N-acetylglucosamine content of the powder were determined by the method of Carbazole reaction[21,26,27] and Boas[28]. The hyaluronic acid complex powder was mainly composed of glucuronic acid, and *N*-acetylglucosamine was of little detected. These results were also identified to that of IR spectrum using 20% KBr (results not shown).

2. Physicochemical properties of the purified hyaluronic acid complex

1) Viscosity

Effect of temperature on viscosity of the purified hyaluronic acid complex was investigated in the range of 10℃ to 90℃(Fig. 3). High viscosity showed at low temperature, but it was decreased gradually by ascending temperature. It was also observed that as temperature was descended from 90℃ to 10℃, its viscosity was increased, however it was not recovered originally.

These results were similar to that of hyaluronic acid from *Streptococcus eqiu* PCI 1988[5, 22] and we assume that low viscosity in high temperature is probably due to the structural deformation of double strand hyaluronic acid complex by heat of high temperature(Fig. 3).

Fig. 4 show effect of pH on the viscosity of purified hyaluronic acid complex. It was very stable at pH 5.0 to 11.0, but unstable at below pH 5.0 and above pH 11.0. This is probably caused by reversible structural change of hyaluronic acid complex at strong alkaline(Fig. 4).

Meanwhile, effect of salts on the viscosity was

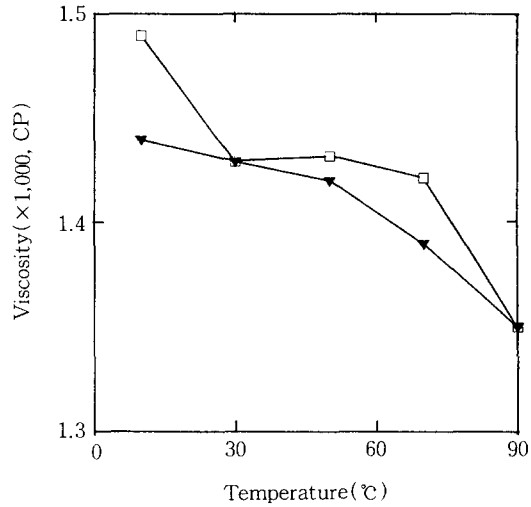

Fig. 3. Effect of temperature on viscosity of 1.0% hyaluronic acid complex solution from *Klebsiella* sp. L-10 NTG 50. □−□ : ascend temperature (10℃ to 90℃), ▼−▼ : descend temperature (90℃ to 10℃).

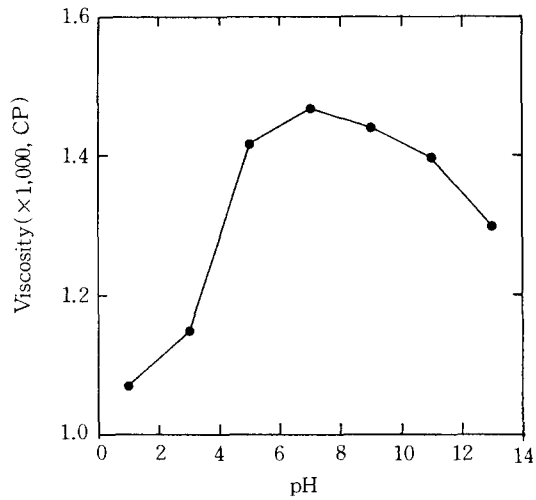

Fig. 4. Effect of pH on viscosity of 1.0% hyaluronic acid complex solution from *Klebsiella* sp. L-10 NTG 50.

Table 2. Rheological properties of purified hyaluronic acid complex from *Klebsiella* sp. L-10 NTG-50

Water content added (%)	Hardness (kg)	Cohesiveness	Adhesiveness(dyn/cm)
30	1.20	1.91	0.62
50	1.00	1.60	0.40

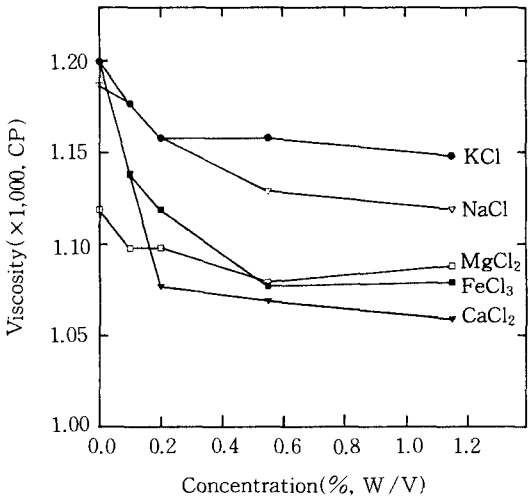

Fig. 5. Effect of various salts concentration on viscosity of 0.3% hyaluronic acid complex solution from *Klebsiella* sp. L-10 NTG 50.

investigated in 3% solution. As shown in Fig 5, increase of salts concentration to 0.6% was decreased the viscosity of purified hyaluronic acid complex(Fig. 5).

2) Rheological properties

Hardness, adhesiveness and cohesiveness of the purified hyaluronic acid complex were calculated from texture profile curve by compression test and presented in Table 2.

Hardness and adhesiveness of the purified hyaluronic acid complex were 1.2 kg, 0.62 at 30% water added, and cohesiveness was 1.91. The hardness and cohesiveness were lower than those of natural starch materials, whereas cohesiveness was higher than theirs.

It was also observed that all of rheological properties were decreased as distilled water added to 50%.

3) Water holding capacity

The purified hyaluronic acid complex was absorbed 6.9ml of distilled water per g.

In conclusion, we could simply purify the hyaluronic acid complex by two-phase extraction system (PEG-K_2HPO_4). Furthermore, its viscosity, cohesiveness and water holding capacity were good. Therefore, we are studying application of the purified hyaluronic acid complex into medical or cosmetic industry.

요 약

Klebsiella sp. L-10 의 *N*-methyl-*N'*-nitro-*N''*-nitrosoguanidine 변이주인 *Klebsiella* sp. L-10 NTG-50 으로부터 생산된 고점성의 히알우론산 복합체를 이상계 추출법으로 정제하여 물리화학적 성질을 조사하였다. 정제된 히알우론산 복합체의 점도는 온도와 염농도가 높아짐에 따라 낮아졌고 pH 5.0 이하와 11.0 이상에서도 매우 낮았다. 정제된 히알우론산 복합체의 굳기(hardness)는 1.20kg, 응집성(cohesiveness)은 1.92, 점착성(adhesiveness)은 0.62 이었다. 또한 보수력은 g 당 6.9ml이었다.

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References

1. Laurent, T. C. and Fraser, J. R. : Hyaluronan, *FASEB. J.*, 2397(1992).
2. Kim, T. S. : Study on development of hyaluronic acid and its fermentative production, *Korea Biochemical Engineering J.*, **4**, 56(1990).
3. Prehm, P. : Release of hyaluronate from eukaryotic cells, *Biochem. J.*, **267**, 185(1990).
4. Brake, J. W. and Thacker, K. : Hyaluronan from *Sreptococcus pyogenes* type 18, U. S. Patent, 4517295 (1985).
5. Chang, L. S. and Jung, K. M. : Production of hyaluronic acidby chemostat culture, *Kor. J. Biotechnol. Bioeng.*, 1, 25(1987).

6. Oh, D. K. : Purification of biosynthesized hyaluronic acid and its medical application, *Kor. J. Biotechnol. Bioeng.*, **11**, 15(1996).
7. Brown, K. K. : Production of hyaluronic acid from *Streptococcus equi* and its mutant, U. S. Patent, 4782046(1988).
8. Acasaca, H., Seto, S., Yanagi, M., Fukushima, S. and Mitsui, T. : Production of hyaluronic acid from *Streptococcus zooepidermicus* #104, *J. Soc. Cosmet. Chem.*, **22**, 35(1988).
9. Jung, K. M. and Jung, K. C. : Recovery method of hyaluronic acid, Kor. Patent, 90-5775(1990).
10. Yoo, T. S. : Production of hyaluronic acid from *Streptococcus zooepidermicus, Kor. J. Biotechnol. Bioeng*, **7**, 112(1992).
11. Holmbeck, S. and Lermer, L. : Separation of hyaluronan oligosaccharides by the use of anion-exchange HPLC : *Carbohydrate Reasearch*, **239**, 239(1993).
12. Markovitz, A., Cifonelli, J. A. and Dorfman, A : The biosynthesis of hyaluronic acid by group A *Streptococcus, J. Biol. Chem.*, **234**, 2343(1959).
13. Stoolmmiller, A. C. and Dorfman, A. : The biosynthesis of hyaluronic acid by *Streptococcus, J. Biol. Chem.*, **244**, 236(1969).
14. Sugahara, K., Schwartz N. B. and Dorfman, A. : Biosynthsis of hyaluronic acid by *Streptococcus, J. Biol. Chem.*, **254**, 6252(1979).
15. Triscott, M. X. and Rijn, van de Rijn. : Solubilization of hyaluronic acid synthetic activity from Streptococcus and its activation with phospholipids, *J. Biol. Chem.*, **261**, 6004(1986).
16. Mausolf, A., J. Jungman, H. Robenek and P. Prehm : Shedding of hyaluronate synthase from *Streptococci., Biochem. J.* **267**, 191(1990).
17. Heldin, P., Asplund, T., Ytterberg, D., Thelin, S. and Laurent, T. C. : Characterization of the molecular mechanism involved in the activation of hyaluronan synthetase by platelet derived growth factor in human mesothelial cells, *Biochem. J.* **283**, 165 (1992).
18. Lansing, M., Lellig, S., Mausolf, A., Martini, I., Crescenzi, F., Oregan, M. and Prehm, P. : Hyaluronate synthase ; cloning and sequencing of the gene from *Streptococcus* sp., *Biochem. J.* **289**, 179 (1993).
19. De angelis, P. L., Papaconstantinou, J. and Weigel, P. H. : Isolation of a *Streptococcus pyogens* gene locus that direct hyluronan biosynthesis in capsular mutants and in Leterologous bacteria, *J. Biol. Chem.*, **268**, 14568(1993).
20. De angelis, P. L., Papaconstantinou, J. and Weigel, P. H. : Molecular cloning, identification and sequence of the hyaluronan synthase gene from group A *Streptococcus pyogens, J. Biol. Chem.*, **268**, 19181 (1993).
21. Lee, H. S., Choi, Y. J. and Lee, J. S. : Production of high viscous hyaluronic acid complex from *Klebsiella* sp. L-10, *J. Natural Sci. Paichai Univ.*, **7**, 59 (1995).
22. Chang, L. S. : *Optimization of reactor for culture of high viscous hyaluronic acid.* Ph. D. thesis Seoul Natl. Univ.(1992).
23. Lee, H. S., Choi, Y. J. and Lee, J. S : Production of high viscous hyaluronic acid complex from *Klebsiella* sp. L-10 NTG-50, *J. Natural Sci. Paichai Univ.*, **8**, 33(1995).
24. Lee, C. H., Chae, S. K. and Lee, J. K. : *Introduction to quality control of food industry*, Yoolim Culture Co., Seoul, p78(1991).
25. Lin, M. J., Humbert, E. S. and Sosulski, F. W. : Certain functional properties of sunflower meal products, *J. Food Sci.*, **37**, 368(1974).
26. Bitter, T. and Muir, H. M. : A modified uronic acid carbazole reaction, *Anal. Biochem.*, **4**, 330(1962).
27. Dische, Z.: A new specific color reaction of hexuronic acids, *J. Biol. Chem.* **167**, 189(1947).
28. Boas, N. F. : Methods for the determination of hexosamines in tissue, *J. Biol. Chem.*, **204**, 553(1953).

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