Effective Production of Hyaluronic Acid Oligosaccharides from Hyaluronic Acid

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The hyaluronidase-mediated degradation of hyaluronic acid to hyaluronic acid oligosaccharides was investigated. The final products of the batch reaction were the tetrasaccharide and hexasaccharide only. In the reaction solution containing an inorganic salt such as NaCl, the yield of hexasaccharide was higher than that of tetrasaccharide. However, in the absence of inorganic salt, the tetrasaccharide was generated preferentially. The yield of each oligosaccharide depended on the nature of the inorganic salt used. In the batch reaction, the product yield was approximately 60%. However, a continuous reaction increased the yield to about 85% within the first 200 h of operation.

Key words: hyaluronic acid, hyaluronidase, hyaluronic acid oligosaccharides, batch reaction, continuous reaction

1. Introduction

Hyaluronic acid is a linear, high molecular mass molecule, composed of the repeating disaccharide unit, β -1, 3-*N*-acetylglucosaminyl β -1, 4-glucuronide. It is an important glycosaminoglycan component found mainly in connective tissues such as cartilage and joints.

There has been renewed interest in the synthesis of hyaluronic acid oligosaccharides since the recent publication of a report linking these molecules of 3 to 12 disaccharide units with the suppression of local tumors [3]. A number of microorganisms are known to possess enzymes which decompose hyaluronic acid. However, the number of the repeating disaccharide unit as final products can vary depending on the origin of the enzyme which was produced by microorganisms. Shimada and Matsumura [1] and Park *et al.* [2] investigated the process by which hyaluronic acid is degraded by hyaluronidase isolated from *Streptomyces* sp. They reported that octasaccharides obtained during the degradation process were the substrates of minimum size for the enzyme, and that tetra- and hexa-saccharides were not degraded further and accumulated as the final products. The molar ratio of tetrasaccharide to hexasaccharide was *ca.* 1 to 1.2.

In this study, we found that the ionic strength of the reaction solution significantly influenced the molar ratio of

the final products. Furthermore, we examined the most effective conditions for producing oligosaccharides in both a continuous and a batch type reaction.

2. Materials and Methods

2.1 Materials

Hyaluronidase derived from *Streptomyces* sp. and sodium hyaluronic acid (average molecular weight of > 800,000) were provided by Amano Enzyme Co. (Nagoya, Japan), and Maruha Co. (Tokyo, Japan), respectively. Protein was removed from the reaction mixture using a Centricut V-10 filter unit (Kurabou Co., Osaka, Japan).

2.2 Batch reaction

The reaction solution of 50 mL was prepared using sodium acetate buffer (0.02 M, pH 6.0) containing NaCl (0.15 M). The hyaluronic acid substrate was dissolved in the buffer at a concentration of 1.0% (w/v). Hyaluronidase was used at a final concentration of 16 TRU/mL; where one TRU (turbidity reducing unit) is defined as the amount of enzyme which causes a 50% reduction in absorbance at 660 nm in 30 min at 60°C. All batch reactions were done at 40°C. At regular time intervals, an aliquot of the reaction solution was sampled and the enzyme removed by centrifugation through a Centricut V-10 filter unit (3,000 $\times$ g for 40 min at 4°C). The recovered filtrate was analyzed by high-performance liquid chromatography (HPLC) using a TSK-Gel DEAE-5PW column (Tosoh Co., Tokyo, Japan)

Fig. 1 Outline of the continuous reaction system.

fitted with a refractive index detector. HPLC was carried out under the following conditions: flow rate, 1.0 mL/min; column temperature, 40°C; eluent composition, 50 mM NaCl. To study the effect of inorganic salts on the enzymatic degradation of hyaluronic acid, KCl, MgCl₂, MgSO₄, FeSO₄ or FeCl₂ (0.15 M each) was used instead of NaCl.

2.3 Continuous reaction

Figure 1 shows an outline of the continuous reaction system [4]. The continuous reaction was performed in a reaction solution of 50 mL at either 40°C or 60°C. The enzyme and residual substrate were kept in the reactor by the membrane (PM10, cut-off molecular mass: 10,000). The working volume in the reactor was kept constant by connecting the liquid surface controller (Level gauge SA-107; Fujiwara Co., Tokyo, Japan) and the peristaltic pump 1. Hyaluronic acid solution (1.0% w/v) in sodium acetate buffer (0.02 M, pH 6.0) and 0.15 M NaCl was kept in the substrate reservoir and fed *via* peristaltic pump 1 to the reactor containing hyaluronidase (final concentration either 16 or 32 TRU/mL). The flow rate at the inlet and outlet of the reactor was 1 mL/h. The reaction was initiated by pumping hyaluronic acid solution into the reactor. The reaction products were transferred to the collector *via* peristaltic pump 2. The concentration of the products was measured by HPLC.

3. Results and Discussion

3.1 Batch reaction

Figure 2 shows the effect of the addition of NaCl to the production of oligosaccharides. When the reaction solution contained 0.15 M NaCl, the concentration of tetrasaccharide was lower than that of hexasaccharide regardless of the reaction period, and the molar ratio of the former to the

Fig. 2 Time course of the hyaluronidase-mediated degradation of hyaluronic acid in the absence or presence of NaCl (0.15 M). The enzyme concentration was 16 TRU/mL and the reaction temperature 40°C. Symbols: ● and ○ represent the concentration of hexasaccharide in the presence or absence of NaCl, respectively; ▲ and △ represent the concentration of tetrasaccharide in the presence or absence of NaCl, respectively.

latter was approximately *ca.* 1 to 1.2–1.3. The other saccharides were not detected during the reaction period. The molar yield of the oligosaccharides was very similar to the result described previously [2]. The yield of both oligosaccharides (tetrasaccharide: 2.2 mg/mL and hexasaccharide: 3.6 mg/mL) was approximately 60%. When the reaction was performed in the absence of NaCl, the ratio of oligosaccharide product was reversed (*i.e.* more tetrasaccharide than hexasaccharide). This interesting result suggests that NaCl in the reaction solution influenced the mechanism of enzymatic degradation of hyaluronic acid. Therefore, the effect of the addition of several inorganic salts on the enzymatic degradation of hyaluronic acid was examined (Table 1). When we substituted NaCl in the reaction solution for other inorganic salts, the yield of hexasaccharide was higher than that of tetrasaccharide. The enzyme activity and the molar yield of tetra- and hexa-saccharide depended on whether inorganic salts were present or not.

Table 1 Concentration and yield of oligosaccharide produced.

Inorganic salts added (0.15 M)	None	NaCl	KCl	MgCl ₂	MgSO ₄	FeSO ₄	FeCl ₂
Tetrasaccharide (g/L)	3.3	2.2	2.4	2.0	1.4	0	0
Hexasaccharide (g/L)	2.8	3.6	4.6	4.4	2.8	1.2	0
Yield (%)	61	58	70	64	42	12	0

3.2 Continuous reaction

In the batch procedure, the reaction appeared to reach equilibrium after about 40 h, and the yield of oligosaccharides was approximately 60%. Therefore, if the oligosaccharide product could be efficiently removed from the reaction solution, further degradation of hyaluronic acid might be expected. We constructed a reactor in which the enzyme was retained by an ultrafiltration membrane, which allowed us to perform a continuous reaction. Figure 3 shows the time course of production of hyaluronic acid oligosaccharides. When the reaction was carried at 40°C using 32 TRU/mL of hyaluronidase, the concentration of the product at the outlet reached a steady state after about 100 h. During a 200-h reaction we obtained *ca.* 1400 mg of oligosaccharide product (560 mg tetrasaccharide and 820 mg hexasaccharide) corresponding to a 70% yield. The steady state can be maintained for approximately 400-h (data not shown), although prolonging the reaction further results in adsorption of residual substrate to the surface of the membrane. However, we detected no enzyme inactivation during the 400-h operation period. When the reaction was carried at 60°C, maintaining other

operational conditions as described above, we obtained *ca.* 1700 mg of oligosaccharide product (760 mg tetrasaccharide and 950 mg hexasaccharide) corresponding to a 85% yield during a 200-h reaction. These results indicate that the yield of product significantly increased at the elevated reaction temperature. However, raising the temperature to increase the yield is technically difficult in a continuous process because of the increased rate of evaporation of the reaction solution.

4. Conclusions

The detectable products from the degradation of hyaluronic acid with the *Streptomyces* hyaluronidase were only hexa- and tetrasaccharides. When the reaction was carried out in the presence of NaCl, more hexasaccharide was produced than tetrasaccharide, but in the absence of NaCl, the product ratio was reversed. A similar trend was also observed using different salts. The observations are of interest in terms of producing these biologically important oligosaccharides although the mechanism of the reaction remains to be solved.

To improve the yield of oligosaccharide a continuous reaction was performed. The oligosaccharides were obtained in high yield (approximately 85%) after 200 h of reaction at 60°C. There was no detectable inactivation of the enzyme during this operation period. Although the continuous process initially generated product efficiently, longer-term reaction (> 400 h) was made difficult by adsorption of residual substrate to the filter.

References

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Fig. 3 Time course of product formation in the continuous reactor. Concentration of hyaluronidase was 32 TRU/mL. The buffer in the reaction solution contained NaCl (0.15 M). The concentration of hyaluronic acid in the reservoir was 10 g/L. The concentration of each oligosaccharide represents the value at the outlet of the reactor. Symbols: ● and ○ represent the concentration of hexasaccharide at 40°C or 60°C of the reaction temperature, respectively; ▲ and △ represent the concentration of tetrasaccharide at 40°C or 60°C of the reaction temperature, respectively.