Methyl Ester of Hyaluronate Is Unable to Stimulate Exolipase Formation by *Serratia marcescens*

KARL-E. JÄGER AND ULRICH K. WINKLER*

Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität, D-4630 Bochum, West Germany

Received for publication 6 July 1979

Hyaluronate stimulated the formation of exolipase by *Serratia marcescens.* This ability was abolished when all carboxyl groups of hyaluronate were methyl esterified. Additional studies suggested that the biological inactivity of esterified hyaluronate should be ascribed to the reduced conformational order of the molecules rather than to their electroneutrality.

The formation of extracellular lipase ("exolipase") by *Serratia marcescens* SM-6 can be stimulated by supplementing the growth medium with certain non-metabolizable polysaccharides (9, 11). All stimulatory polysaccharides presently known are distinguished by a high degree of structural order, enabling interstrand molecular interactions. One assumes that stimulatory polysaccharides act by detaching exolipase molecules from temporary storage sites on the cell surface of *S. marcescens* (11). Potassium hyaluronate is one of the stimulatory polysaccharides (11). This paper describes the methyl esterification of hyaluronate and some physical and biological properties of the product. It was expected that methyl esterification would reduce the conformational order of hyaluronate and, consequently, its exolipase-stimulating ability.

Hyaluronic acid (HA) is a linear polymer with repeating β-1,4-linked disaccharide units. Each unit is composed of d-glucuronic acid and N-acetyl-d-glucosamine in β-1,3 linkage. Throughout this study we used a preparation of K-HA of high molecular weight (ex-umbilical cord; Serva, Heidelberg, Germany). Its chemical analysis by various methods (3, 4, 8) revealed a 1:1 ratio of glucuronic acid and hexosamines and a content of 3.3% protein. According to the manufacturer, the preparation contained less than 3% of chondroitin sulfate. A 1-mg amount of K-HA contained 3.5 µg of Ca²⁺ and 86.5 µg of K⁺ as detected by atomic absorption spectroscopy (kindly performed by E. Jackwerth, Bochum).

Unmodified K-HA stimulated the formation of exolipase by *S. marcescens* W1270 in a concentration-dependent fashion (Fig. 1). This particular strain produces exolipase only in the presence of exogenously applied cyclic AMP (10). The data shown in Fig. 1 were obtained from experiments performed under chemically defined conditions (see legend to Fig. 1). This

![Graph](http://jb.asm.org/)  
**Fig. 1.** cAMP-induced formation of exolipase by *S. marcescens* W1270 (10) as a function of the concentration of K-HA and CaCl₂, respectively. The bacteria were grown in supplemented M9 glucose medium to a cell density of 1 × 10⁹ per ml (11) and then suspended in 53 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM KCl. The formation of exolipase was induced by incubating the cells (3 × 10⁹ per ml) for 15 min at 30°C with 2 mM cAMP and various concentrations of K-HA (△) or CaCl₂ (●). The exolipase was assayed with p-nitrophenylpalmitate as substrate (11); one enzyme unit is defined as 1 nmol of p-nitrophenol ml⁻¹ min⁻¹. The exolipase activity found in the absence of HA or CaCl₂ was subtracted from all other enzyme activities measured. The background was 10 enzyme units.
allowed us to conclude that the exolipase stimulation by K-HA is unlikely to be a masked effect of the potassium and calcium ions present in every hyaluronate preparation. It was shown earlier (11) that neither chondroitin sulfate nor the monomeric subunits of hyaluronate are able to stimulate the formation of exolipase.

The preparation of the carboxyl methyl esters of the glucuronic acid residues was performed as follows. An aqueous solution of K-HA (0.03%) was passed through a Dowex 50 WX8 column (1 by 4 cm). The free HA obtained was freeze-dried. After the addition of a few drops of diethyl ether to the dry material (50 mg), the polymer was esterified at 0°C with an ether solution of freshly prepared diazomethane. The molar ratio of diazomethane to disaccharide unit was about 8:1. After 150 min, the ether and excess of diazomethane were removed by distillation under reduced pressure. The dry material was dissolved in distilled water and freeze-dried.

An aqueous solution of methyl esterified hyaluronate (HA\textsubscript{met}) was nearly neutral (Table 1), indicating that most, if not all, of the original carboxyl groups had been esterified. This conclusion was supported by chemical analysis (13) of HA\textsubscript{met}, which revealed 1.15 methyl groups per glucuronic acid residue. Furthermore, the \textsuperscript{13}C nuclear magnetic resonance spectrum of HA\textsubscript{met} (20 mg/ml in D\textsubscript{2}O; see reference 5) showed methyl carbon atoms at the carboxyl groups. The spectra were recorded with a Bruker WH-90 instrument.

Methyl esterified hyaluronate was unable to stimulate the formation of exolipase (Table 1). This biological inactivity of HA\textsubscript{met} should be ascribed to its reduced conformational order rather than to its electroneutrality. The reasons for this assumption are the following. (i) The exolipase-stimulating ability is not restricted to polyanionic polysaccharides, as shown for example by laminaran (11). Therefore, the irreversible neutralization of the carboxyl groups of hyaluronate by esterification per se should not necessarily have abolished its exolipase-stimulating ability. (ii) In the presence of various metal ions, HA molecules form a mesh of closely packed antiparallel double helices; the carboxyl groups are placed toward the center of each double helix and help to stabilize the overall structure (1, 6, 7, 12). Based on this model, the esterification of the carboxyl groups of hyaluronate as reported in this paper should distort or even destroy the helical structure of K-HA. The low viscosity of solutions of HA\textsubscript{met} compared with that of K-HA solutions (Table 1) is in full agreement with this expectation. When the viscosity of K-HA solutions was reduced by treatment with hyaluronidase (EC 3.2.1.35) instead of diazomethane, the biological activity of hyaluronate remained constant up to a residual viscosity of about 15% (data not shown). Therefore decrease of viscosity itself is not obligatorily accompanied by a decline of the biological activity of K-HA.

It was recently observed that methyl esterification of oligomers of HA abolished their ability to interact with bovine nasal cartilage proteoglycan (2). This result seems to parallel our findings.

We are greatly indebted to H. Hemetsberger, University of Bochum, for his valuable advice during the preparation of

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Pretreatment of K-HA</th>
<th>pH\textsuperscript{a}</th>
<th>Cinematic viscosity\textsuperscript{b} (\eta (cS))</th>
<th>Exolipase-stimulating factor\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>6.4</td>
<td>6.21</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Desalted</td>
<td>2.5</td>
<td>0.98</td>
<td>Nd\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>(Dowex 50 WX8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Desalted and reneutralized</td>
<td>6.4</td>
<td>1.20</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Desalted and methyl esterified</td>
<td>6.5</td>
<td>0.88</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} For pH measurements a Knick pH meter type pH27 was used. All samples were dissolved in distilled water (4 mg/ml).

\textsuperscript{b} Viscosimetric measurements were performed with a Micro-KPG-Ubbelohde viscosimeter (capillary diameter, 0.32 mm) at a temperature of 25 ± 0.1°C. Samples no. 1, 2, and 4 were dissolved at a concentration of 0.5 mg/ml in 63 mM Tris-hydrochloride buffer (pH 7.0) containing 50 mM KCl. Sample no. 3 was dissolved in distilled water, dialyzed against 0.1 mM KOH for 48 h, freeze-dried, and then redissolved as samples no. 1, 2, and 4.

\textsuperscript{c} The exolipase-stimulating activity of the various samples was measured as described in the legend to Fig. 1, but the incubation time was 30 instead of 15 min and the final concentration of K-HA or its derivatives was 2 mg/ml. The exolipase activity in the absence of hyaluronate or its derivatives was usually 20 nmol ml\textsuperscript{-1} min\textsuperscript{-1}. (Nd) Not determined.
diazomethane and its use for the esterification, and to W. Dietrich, University of Bochum, for recording the $^1$C nuclear magnetic resonance spectra.

**LITERATURE CITED**


