

Proceeding Paper

In Vitro Biodegradability of Phosphorylated Hyaluronic Acid by Testicular Hyaluronidase [†]

Elvira A. Khaybrakhmanova, Stanislav V. Kozyrev and Irina Yu. Ponedel'kina *

Institute of Petrochemistry and Catalysis, Ufa Federal Research Center, Russian Academy of Sciences, 141, Prospekt Oktyabrya, 450075 Ufa, Russia; email1@email.com (E.A.K.); email2@email.com (S.V.K.)

* Correspondence: ponedelkina@rambler.ru

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Abstract: One of the most important characteristics that is of great importance for the development of biomaterials based on hyaluronic acid (HA) and its chemically modified derivatives is resistance to hyaluronidases (HA-ase) responsible for the degradation of HA macromolecules. In this work, samples of phosphorylated (HA-P) low and high molecular weight HA (43 kDa and 0.5–0.7 MDa) were obtained by conventional stirring of HA/P₂O₅ mixtures at different molar ratios (1:0.2, 1:0.5, 1:1 and 1:2) and exposed to bovine testicular HA-ase (60 or 600 ME/mL, [HA] = 4 mg/mL of citrate buffer, pH 6.3, 16–18 h). It was found that biodegradability of the HA-P samples obtained at the ratio of 1:0.2, 1: 0.5, and 1:1 was at the level of native HA or even exceeded the HA biodegradability. Only samples obtained at a HA/P₂O₅ ratio of 1:2 showed resistance to HA-ase: their biodegradability was 13–54% lower compared to that of native HA.

Keywords: keyword 1; keyword 2; keyword 3 (List three to ten pertinent keywords specific to the article yet reasonably common within the subject discipline.)

1. Introduction

The biodegradability of hyaluronic acid (HA) and its chemically modified derivatives under the action of hyaluronidases (HA-ases) is one of the most important characteristics of biomaterials developed on the HA basis. Any undertaken chemical modification of HA can lead to both a decrease and an increase in biodegradability. For example, the cross-linking of HA [1] or C6-oxidation at the *N*-acetyl-D-glucosamine unit [2] reduce its biodegradability, while the introduction of vitamin substances such as nicotinic acid (vitamin PP) into the structure activates the biodegradability of HA macromolecules [3].

Recently, two HA samples with different molecular weights (43 kDa and 0.5–0.7 MDa) were successfully phosphorylated via simple stirring with P₂O₅ for 2 h at room temperature. Depending on the HA/P₂O₅ ratio, the HA-P derivatives contained from 0.30 to 6.25% P and were characterized by various types of covalently bound phosphate residues, from mono- and diphosphates to polyphosphate (PP) [4]. We hypothesized that these structural features may influence the biodegradability of HA-R in unexpected ways. Therefore, the purpose of this work was to test HA-P samples for resistance to bovine testicular HA-ase.

2. Materials and Methods

The samples of low molecular weight (LMW, 43 kDa, Leko Style, St.-Petersburg, Russia) and high molecular weight (HMW, 0.5–0.7 MDa, Contipro, Czech Republic) HA were used. Phosphorous (V) oxide was purchased from Acros Organics. D₂O was bought from Eurisotop. Other chemicals were of analytical reagent grade. Bovine testicular hyaluronidase (HA-ase, pharmacopoeial drug Lidase) was obtained from Microgen (Russia) and used without further purification.

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The biodegradability of the samples was determined as follows. To the 0.9 mL citrate buffer (30 mmol citric acid, 150 mmol Na₂HPO₄, 150 mmol NaCl) containing 0.01 mmol LMW HA or HMW HA or corresponding HA-P 1–8, HA-se solution was added (60 or 600 IU in the 0.1 mL citrate buffer). The samples were incubated for 16–18 h at 37 °C then 4 mL of the mixture MeOH/Et₂O (3:1) as a precipitant was added to each solution. The resulting precipitates were separated by centrifugation. Supernatants were evaporated, the dry residues were dissolved in 10 mL water and solutions of low-molecular weight products of enzymatic degradation were analyzed for the content of D-glucuronic acid using the carbazole method of Dische in the modification of Bitter and Muir.

3. Results and Discussion

Main text paragraph (M_Text). Phosphorylated samples LMW HA-P 1–4 and HMW HA-P 5–8 were obtained according to the method described in [4]. The reaction scheme for HA phosphorylation with P₂O₅ and the reaction products are shown in Figure 1.

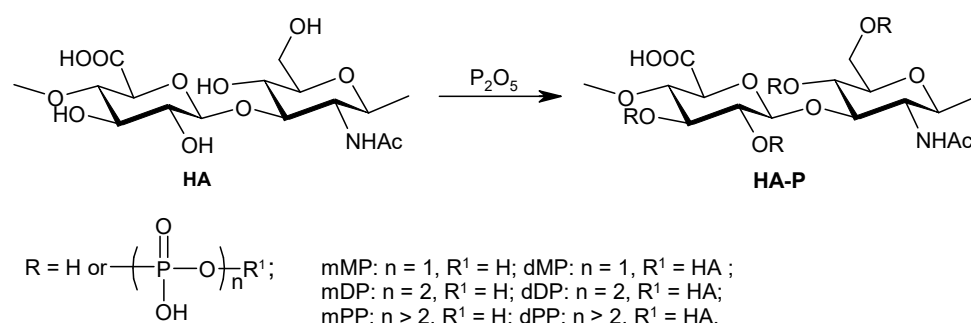


Figure 1. Reaction scheme for HA phosphorylation with formation of all possible phosphorylated HA derivatives.

The total P content in the samples and its distribution in the form of disubstituted monophosphates (dMP), αP in disubstituted diphosphates and polyphosphates (dDP + dPP), and middle $-(\text{P})_n-$ in dPP depending on the HA/P₂O₅ ratio are given in Table 1 (cm. [4]).

The biodegradability of native LMW HA and HMW HA and phosphorylated HA-P samples was determined by the amount of low-molecular-weight products formed during the cleavage of HA by HA-ase (60 or 600 ME/mL, [HA] = 4 mg/mL of citrate buffer, pH 6.3, 16–18 h) and not precipitated by the methyl alcohol/diethyl ether mixture (3:1) [2]. Relative biodegradability (RB) of HA-P samples was calculated relative to the biodegradability of the corresponding LMW HA and HMW HA.

Table 1. Characteristics of LMW HA-P (entries 1–4) and HMW HA-P (entries 5–8) samples and relative biodegradability of those depending on the HA/P₂O₅ ratio.

No.	HA/P ₂ O ₅	Total P, % wt.	P in dMP; αP in dDP + dPP; $-(\text{P})_n-$ in dPP, % wt. *	RB, % of that for HA ** (HA-ase 60 IU/mL) ***		RB, % of that for HA ** (HA-ase 600 IU/mL) ***	
				1st Series	2nd Series	1st Series	2nd Series
entry 1	1:0.2	1.39	0.63; 0.60; 0.16	99	104	92	88
entry 2	1:0.5	1.28	0.07; 1.00; 0.21	93	98	84	108
entry 3	1:1	1.74	0.06; 1.34; 0.30	95	107	92	85
entry 4	1:2	6.25	0.10, 5.60, 0.30	92	66	87	68
entry 5	1:0.2	0.30	not determined	92	148	90	120
entry 6	1:0.5	0.75	not determined	97	158	85	88
entry 7	1:1	2.55	0.07; 0.57; 1.91	90	153	78	101
entry 8	1:2	2.25	0; 0.55; 1.70	71	55	87	46

* The composition of phosphate residues was determined using ³¹P NMR spectroscopy [4]. ** The biodegradability of native LMW HA and HMW HA was assumed to be 100%. ** The

biodegradability of samples was determined in two parallel series of experiments. The first and second series of experiments on the enzymatic degradation of samples by bovine testicular HA-ase differed in the date of manufacture of Lidase (expiration date until November 2024).

The Table 1 shows that RB in most experiments of the first series was practically independent of the characteristics of the samples or the conditions of enzymatic reactions and was at the level of unmodified LMW HA and HMW HA. A slight trend toward RB decreasing, 13–22%, was observed in entries 2, 4 and 6–8 when using HA-ase concentration of 600 IU/mL. Only one sample 8, prepared at a HMW HA/P₂O₅ ratio of 1:2, was more resistant (~30%) to degradation by HA-ase (60 IU/mL) compared to HMW HA.

In the second series of experiments, the RB of low phosphorylated LMW HA-P samples (HA/P₂O₅ 1:0.2, 1:0.5, and 1:1) was at the level of unmodified LMW HA at both HA-ase concentrations. A decrease in RB by 32–34% (60 and 600 IU/mL of HA-ase) was observed only for the LMW HA-P sample obtained at HA/P₂O₅ 1:2 (entry 4, P 6.25% wt.).

Interesting results were obtained for samples HMW HA-P 5–7 (at 60 IU/mL of HA-ase). Compared to native HMW HA, their RB was one and a half times higher. In this regard, it should be noted that these are not random results. Single experiments we did earlier using Lidase from a different batch showed for the same samples HMW HA-P a threefold excess of RB relative to unphosphorylated HA. These facts have not yet received an explanation. But it can be assumed that phosphorylated HA derivatives with a P content of no more than 2% may have vitamin-like properties. At a HA-ase concentration of 600 IU/mL, the biodegradability of the same samples 5–7 was on average slightly higher than that of HMW HA. A significant decrease in RB, up to 55% (at 60 IU/mL) and 46 (at 600 IU/mL), was observed only for the HMW HA-P 8 sample (HA/P₂O₅ 1:2).

Thus, the first experimental studies of the biodegradability of phosphorylated HA derivatives by bovine testicular HA-ase showed that test results may depend on both HA-ase activity and sample characteristics. A more or less significant decrease in biodegradability (or increase in resistance to HA-ase) was observed for samples obtained at a HA/P₂O₅ ratio of 1:2.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Prestwich, G.D.; Marecak, D.M.; Marecek, J.F.; Vercruyse, K.P.; Ziebell, M.R. Controlled chemical modification of hyaluronic acid: Synthesis, applications, and biodegradation of hydrazide derivatives. *J. Control. Release* **1998**, *53*, 93–103. [https://doi.org/10.1016/S0168-3659\(97\)00242-3](https://doi.org/10.1016/S0168-3659(97)00242-3).
2. Ponedel’kina, I.Y.; Odinkov, V.N.; Saitgalina, E.A.; Dzhemilev, U.M. The in vitro resistance of oxidized hyaluronic acid to testicular hyaluronidase. *Dokl. Biochem. Biophys.* **2007**, *417*, 341–342. <https://doi.org/10.1134/S1607672907060142>.
3. Ponedel’kina, I.Y.; Lukina, E.S.; Khaibrakhmanova, E.A.; Sal’nikova, E.V.; Odinkov, V.N. Conjugates of hyaluronic and carboxy-hyaluronic acids and their in vitro biodegradability upon the action of testicular hyaluronidase. *Russ. Chem. Bull.* **2011**, *60*, 754–759. <https://doi.org/10.1007/s11172-011-0116-9>.
4. Khaybrakhmanova, E.A.; Kozyrev, S.V.; Tyumkina, T.V.; Ponedel’kina, I.Y. Phosphorylation of Hyaluronic Acid. *Chem. Proc.* **2022**, *12*, 39. <https://doi.org/10.3390/ecsoc-26-13535>.

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