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Research paper

Chemical modification of hyaluronidase regulates its inhibition by heparin

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Abstract

Chemical modification of surface amino groups of bovine testicular hyaluronidase with aldehyde dextran was conducted. It was found that with the increase of modification degree of hyaluronidase amino groups the value of residual enzymatic catalytic activity is decreased rather monotonously. It turned out that the value of inhibition of enzyme activity by heparin considerably depends on modification degree of enzyme. This dependence is of a threshold character. Sharp conformational changes in the enzyme occurring at 70–90% degree of its modification considerably lowers heparin inhibition. The higher the degree of hyaluronidase modification, the weaker its inhibition by heparin. More completely/deeply modified derivatives of hyaluronidase (modification degree 96–100%) are practically not inhibited by heparin. Thus, chemical conjugation of hyaluronidase with aldehyde dextran regulates the value of enzyme inhibition by heparin. Hyaluronidase modification becomes an informative tool to study the mechanism of inhibition of its enzyme activity and an efficient means for the development of new therapeutic preparations improving tissue permeability during cardiovascular injuries.

Keywords: Testicular hyaluronidase; Dextran; Chemical modification; Heparin inhibition

1. Introduction

Polysaccharide structures are a part of many biologically active agents, regulating biosystem functioning. Thus, heparin, serving as a template/matrix for thrombin interaction with antithrombin III, considerably increases the efficacy of thrombin inhibition in blood flow [1]. In glycoproteins a carbohydrate part of natural origin, like in tissue plasminogen activator [2], or synthetic origin, like in mono- or polysaccharide modified superoxide dismutase [3], determines clearance of such conjugates by liver cells. High molecular hyaluronic acid (molecular mass more than 750 kDa) can form a complex with fibrinogen at the sites of tissue injury, promoting the increase of blood viscosity and have an influence on the state of microcirculation and blood pressure [4,5]. Such unfavorable effects in general slow down fibrinolysis and demand the presence of hyaluronidase activity. However, the carrier of such activity - hyaluronidase enzyme is inhibited in the organism by macromolecules of sulfated mucopolysaccharides [6]. Hyaluronidase inhibition is not studied well enough and only the data on general integral effects of this interaction is available. Its consequences may be negative as in the case mentioned above, when thrombus formation is accelerated and thrombus structure is changed [4], as well as positive upon inhibition of hyaluronidase – 'spreading factor' – by flavonoids which are contained in such agents of 'folk medicine' as some plant extracts [7] serving as an antidote during poisonous bites. However, thorough study of the regulation of catalytic activity of hyaluronidase has not yet been conducted.

Hyaluronidase is a globular enzyme of endoglycosidase action, which can depolymerise hyaluronic acid in the organism, decreasing its viscosity and increasing tissue permeability. Hyaluronidase is used in clinic for local application (subcutaneous injections) during the treatment of a disease of joints, in dermatology and ophthalmology (with the aim of formation of a thinner scar). It was supposed that hyaluronidase may prove to be useful for decreasing myocardial infarction size, since it was demonstrated in biomedical studies on animals [8]. However, clinical investigations (9450 patients, from 1978 to 1983) did not reveal any significant difference in the action of intravenously administered native hyaluronidase compared with the indexes of placebo group (serum albumin) of patients [8]. Possible reasons for such conclusion are connected with later beginning of therapy (more than 9 h from the onset of symptoms) [9], organization and compiling protocol of

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trials [10] and inhibition of native testicular hyaluronidase by heparin [11]. However, the urgency of the search for agents, decreasing myocardium infarct size in patients does not become less because such an approach promotes a decrease of the terms of disease and a decrease of mortality. Therefore hyaluronidase of leeches was suggested for this role [10], which is not inhibited by heparin. It was also shown, that combined administration of hyaluronidase (2500 IU/kg) with urokinase (40 000 IU/kg) into rats with myocardial infarction significantly decreased mortality data, when the lethal index for combination of preparations was 12.5% against 29.5% for urokinase [12]. Above all, it was found by the method of positron emission tomography that after successful thrombolysis of occluded vessels, tissue permeability in patients with acute myocardial infarction deteriorates because of microcirculation disturbances induced by ischemia and reperfusion [13]. Functional recovery of myocardium is observed only during adequate restoration of tissue permeability/tissue flow. Thus, supplementary therapy, which improves microcirculation, is needed for recovery. Hyaluronidase activity might be useful for that. These data show the importance of searching for the ways to regulate a hyaluronidase inhibition in biosystems.

We have earlier demonstrated that as the result of covalent coupling of hyaluronidase (via surface functional amino groups) to aldehyde dextran the enzyme increases its stability under physiological conditions and possesses a pronounced inhibitory action on the progress of experimental silicosis in rats [14]. Under these conditions the native enzyme did not exert a therapeutic effect. This modification weakened action of inhibitors and promoted successful combined application of aldehyde dextran modified forms of hyaluronidase and superoxide dismutase [15] showing the way for the development of efficient courses of enzyme therapy. Chemical modification of hyaluronidase makes it suitable for systemic inhalation administration [14], which may prove to be effective for the administration into the blood flow also. Therefore consecutive study of chemical modification of hyaluronidase by its surface amino groups promises to elucidate influence on enzyme inhibition by heparin. As a modifier we used aldehyde dextran, which is already applied in clinics [14,15].

The aim of the present study was to search for the ways of regulation for heparin inhibition of hyaluronidase by chemical modification of enzyme with aldehyde dextran. For that we have conducted consecutive modification of enzyme amino groups and studied the effect of degree of hyaluronidase modification on the value of heparin inhibition of this biocatalyst.

2. Materials and methods

2.1. Materials

We used bovine testicular hyaluronidase (EC 3.2.1.35) (Immunepreparation, Ufa, Russia). The commercial name of the enzyme preparation is lidase®. The enzyme was preliminary purified on sephadex column G-100 (Pharmacia, Uppsala, Sweden). Position of gelchromatography peaks of hyaluronidase activity and protein absorbtion (280 nm) corresponds to molecular mass of 60–62 kDa, according to the data for pure bovine testicular hyaluronidase [16]. This fraction (of mentioned molecular mass) was used in the present study, specific activity of isolated hyaluronidase was 2000 IU/mg protein. In the study we have also used dextran of molecular mass 35–50 kDa (Nutritional Biochemical Corp., Cleveland, OH). Potassium salt of hyaluronic acid (molecular mass 700–800 kDa) from human umbilical cord, trinitrobenzenesulfonic acid (TNBS), sodium periodate, heparin sodium salt (of bovine origin), sodium borhydride, sodium chloride, sodium dodecylsulfate were obtained from Sigma, St. Louis, MO. The rest of the reagents were of domestic production having analytical degree of purity.

2.2. Obtaining of aldehyde dextran

Aldehyde dextran obtaining was conducted by the method of partial periodate oxidation of dextran [17]. According to iodometry an obtained aldehyde dextran contained 20–22 aldehyde groups per hundred glycoside units in polymer chain.

2.3. Protein determination

Protein content in preparations was determined by the method of Bradford [18] with bovine serum albumin as a standard.

2.4. Hyaluronidase modification with aldehyde dextran

Covalent modification of hyaluronidase with aldehyde dextran was performed using a previously applied scheme [14]. Hyaluronidase was incubated with aldehyde dextran in the solution of 0.1 M phosphate buffer containing 0.15 M sodium chloride, pH 8.3 at 4°C. Hyaluronidase concentration in incubation mixture is 10 μM, that of aldehyde dextran 20–200 μM. Incubation time varied from 30 min to 18 h. After that we reduced the formed Shiff bases by sodium borhydride (10 mg/ml) in a cold room for 30–40 min and the obtained adducts were isolated by ultrafiltration at Amicon, USA with XM-100 membrane, and then lyophilized. We have shown the same kinetic ways of obtaining modified forms of hyaluronidase with a similar degree of modification due to variance of either aldehyde dextran concentration in reaction mixture, or incubation time of the latter.

2.5. Modification degree

Measurement of modification degree of hyaluronidase was achieved by the titration of enzyme surface amino groups by trinitrobenzenesulfonic acid using a modified

method [19]. Upon covalent interaction of protein amino groups with aldehyde groups of dextran polymer the number of the first (and the number of the second) is decreased. One may judge the increase in modification degree of hyaluronidase by the decrease in the number of titrated enzyme amino groups. Data of native enzyme (0% for modification degree) was assumed as initial index, complete absence of titrated amino groups of hyaluronidase means 100% degree of its modification.

2.6. Hyaluronidase activity

Determination of enzyme activity of hyaluronidase was conduced viscosimetrically according to indicated recommendations [20]. For this, 1 ml of 0.02 wt.% of potassium hyaluronate in 0.1 M phosphate buffer containing 0.15 M sodium chloride, pH 5.45 in Ostwald viscosimeter B-434 (USA) was placed into a thermostat at 37°C. After 5 min of incubation 10 μl solution of native or modified hyaluronidase in the same buffer solution (concentration by protein 0.2 mg/ml) was added into the viscosimeter and started count of reaction time (t) of the enzyme with substrate. Time of outlfow (τ) of reaction mixture from reservoir of viscosimeter was determined periodically for 15–20 min. On the basis of these data (set of parameters) we calculated the value of relative viscosity, which is a ratio of the outflow time of reaction mixture to the outflow time of the substrate buffer solution without enzyme. The slope of the direct line in the plot of the reverse logarithm of relative viscosity against time of measurement ($t + (\tau/2)$) is proportional to the rate of enzyme reaction [14]. This slope (tangent) gives the relative value of the destruction rate of hyaluronic acid upon enzyme action of hyaluronidase. One international unit (IU) of hyaluronidase activity is the amount of enzyme that will cause the same turbidity reduction as 1.0 unit of International Standard preparation [21]. Comparison of relative values of the destruction rate of hyaluronic acid under the action of studied preparations and standard allows (in the given interval of protein concentrations [20]) the determination of absolute value of hyaluronidase activity in IU.

2.7. Hyaluronidase inhibition by heparin

Inhibition of hyaluronidase activity by heparin was studied in respect to enzyme activity retained after enzyme incubation (by protein 0.2 mg/ml) with heparin (2 – 200 mg/ml) at pH 7.5 (0.02 M phosphate buffer with 0.15 M sodium chloride), 37°C during various time intervals when samples were taken (20 μl) and their enzyme activity was determined as described above.

2.8. Fluorescence measurements

Spectrofluorometric study of hyaluronidase derivatives was conduced on spectrofluorometer Hitachi F-4010 (Japan) at excitation wavelength 280 nm, emission wavelength 348 nm and upon similar protein concentrations in cuvette of 0.2 mg/ml. This value is within the concentration interval (0.05–2.0 mg/ml) where the intensity of protein fluorescence is proportional to the hyaluronidase concentration. Measurement of the intensity value of intrinsic fluorescence of the enzyme was conducted at pH 7.5 (0.02 M phosphate buffer containing 0.15 M sodium chloride) and room temperature. Heparin, salts of buffer solutions do not exhibit intrinsic fluorescence under these conditions.

Incubation time of hyaluronidase with heparin did not considerably effect the intensity value of intrinsic fluorescence of enzyme derivatives used upon similar protein concentrations of samples in cuvette (0.2 mg/ml). Therefore, intensity of intrinsic fluorescence of hyaluronidase derivatives was determined (as indicated above) after 30 min incubation with heparin, creating its different incubation concentrations (2–200 mg/ml) in solution of 0.02 M phosphate buffer containing 0.15 M sodium chloride, pH 7.5 and room temperature (heparin titration).

2.9. Electrophoresis

Electrophoresis separation of hyaluronidase derivatives was conducted in 5–20% polyacrylamide gel in the presence of sodium dodecyl sulfate [22]. Data of denaturating electrophoresis indicate covalent modification of hyaluronidase by aldehyde dextran and absence of significant amounts of intermolecular cross-linked enzyme aggregates.

3. Results and discussion

3.1. Modification decreases heparin inhibition of hyaluronidase

Native hyaluronidase is rather effectively inhibited by heparin (Fig. 1A). With the increase of ratio of weight concentrations of heparin:hyaluronidase, the enzyme inhibition is also increased. After a 30 min incubation with heparin it was noticed that increased concentrations of inhibitor considerably decrease hyaluronidase activity (Fig. 1B). To compare inhibitory action of heparin on the activity of aldehyde dextran treated derivatives of hyaluronidase (with different degree of enzyme amino group modification), we have chosen suitable conditions, which provide noticeable inhibition of native biocatalyst under concentration of reagents close to physiological (curve 3, Fig. 1A). It turned out that hyaluronidase modification decreased value of heparin inhibition (Fig. 2A). When modification degree of hyaluronidase increased, inhibition value was also considerably decreased. Derivative with a high (nearly complete) degree of amino group modification (96–98% from the index of native enzyme) exhibited more stable catalytic activity in the given comparison. Comparison of the values of first-order rate constant for inactivation of hyaluronidase preparations obtained by linearization of curves from Fig. 2A in the plot of logarithm of enzyme

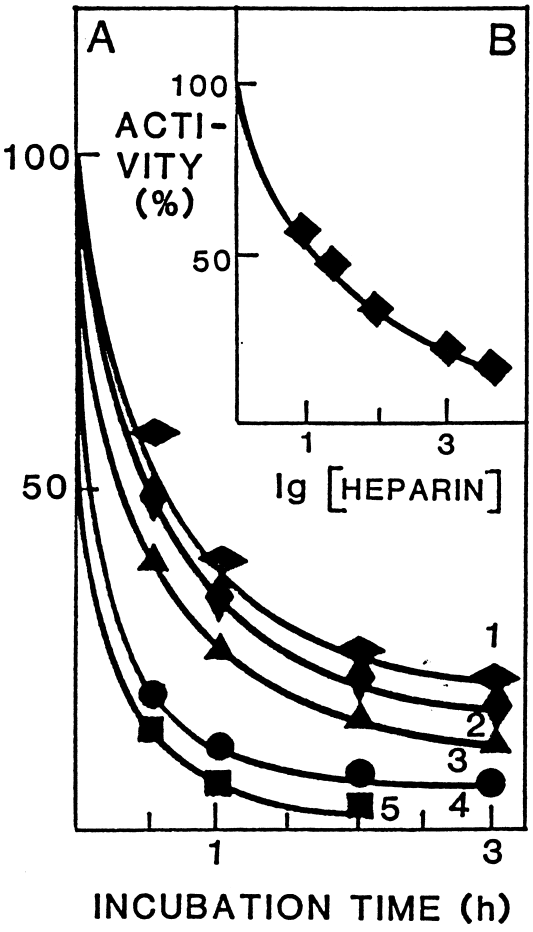

Fig. 1. Heparin effect on enzyme activity of hyaluronidase. (A) Dependence of activity of native hyaluronidase on the time of enzyme incubation with heparin at various ratios of weight concentrations (mg/ml) of hyaluronidase and heparin (respectively). (1) 1:15; (2) 1:25; (3) 1:100; (4) 1:1000; (5) 1:5000. (B) Inhibition of native hyaluronidase activity after half-hour incubation of enzyme with various weight concentration of heparin.

activity against the incubation time (in the interval of 0.5–2 h) shows threshold character of stabilization curve of hyaluronidase activity (Fig. 2B). At more than 70% of degree of hyaluronidase modification, it has developed a considerable effect of stabilization of its activity against inhibitory action of heparin. At 98% degree of hyaluronidase modification there is the observed effect of stabilization, which exceeds more than 15 times indexes for native enzyme (Fig. 2B).

3.2. Regulation of hyaluronidase inhibition by its modification

It should be pointed out that the initial binding of hyaluronidase to aldehyde dextran (with high residual enzyme activity of 90–100%) leads to an increase of biocatalyst thermostability [14]. It shows a certain strengthening of protein structure (against thermoinactivation) as a result of its chemical modification. With the increase of degree of

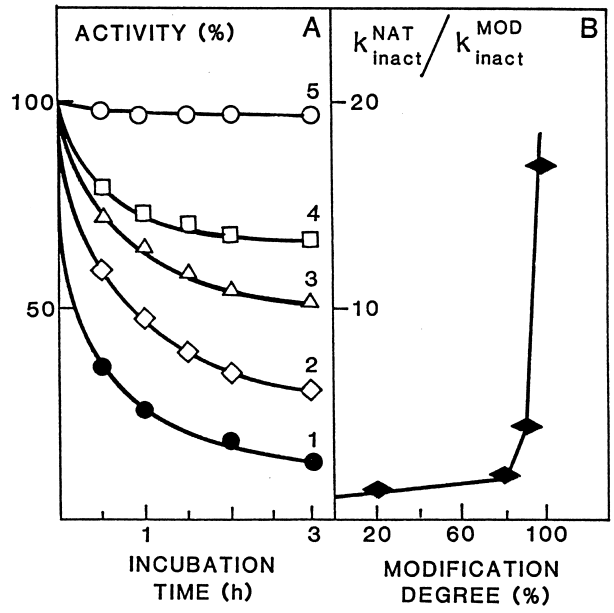

Fig. 2. Effect of hyaluronidase modification on inhibition of its enzyme activity by heparin. (A) Dependence of residual activity of hyaluronidase derivatives after incubation with heparin at ratio of weight concentrations (mg/ml) enzyme and heparin as 1:100, where (1) native hyaluronidase and its modified aldehyde dextran derivatives with modification degree: (2) 20%, (3) 80%, (4) 90%, (5) 98%; (B) stabilization effect of hyaluronidase activity (presented in the form of ratio of the first-order rate constants for inactivation of native and aldehyde dextran modified hyaluronidase) against heparin inhibition depending on the degree of enzyme modification.

hyaluronidase modification the value of residual activity of modified derivatives is decreased (Fig. 3A). In this case the residual activity is determined as the activity of enzyme after its modification (from initial level). The rather monotonous character of this dependence indicated increased changes induced into native protein structure upon increase of the degree of its modification by aldehyde dextran.

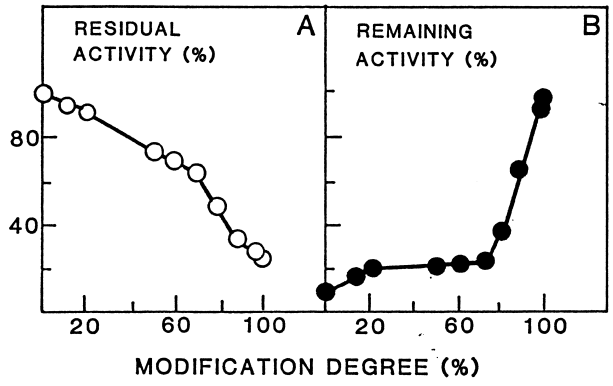

Fig. 3. Dependence of hyaluronidase activity on the degree of its modification with aldehyde dextran. (A) Influence of modification degree of hyaluronidase on residual specific activity of aldehyde dextran modified derivatives of enzyme; (B) remaining specific activity of hyaluronidase derivatives after 1 h incubation with heparin at ratio of weight concentrations (mg/ml) of enzyme and heparin as 1:1000 depending on the degree of biocatalyst modification.

However, evaluation of remaining hyaluronidase activity after 1 h incubation of enzyme derivatives with 1000-fold excess of heparin showed that the inhibition value did not change monotonously, but considerably reduced upon modification degree more than 70% (Fig. 3B). It should be noted that the remaining activity implies the activity of enzyme derivatives in the presence of heparin from their initial residual activity in the absence of heparin, respectively. The most completely/deeply modified hyaluronidase derivatives (98–100%) were not practically inhibited by heparin. These data very clearly show the possibility of regulating heparin inhibition of hyaluronidase depending on the degree of chemical modification of its surface amino groups. It is likely that changes introduced by chemical modification into the native structure of biocatalysts lie at the basis of this effect.

3.3. Stabilizing conformational changes of hyaluronidase induced by its modification

It was noted earlier that consecutive modification of chymotrypsin with the increase of the degree of its amino group modification leads to sharp changes in enzyme thermostability [23]. Modification of hard-to-reach amino groups (with modification degree more than 70%) induces maximal noticeable effects with considerable conformation changes induced by chemical modification. Similarity of obtained data with the results of the mentioned study [23] stipulated the performance of spectrofluorometric study of various hyaluronidase derivatives with different degrees of modification from 0 to 98 %. The intensity index of intrinsic fluorescence of protein is a sensitive test of changing its structural organization. It was established that the relative intensity of intrinsic fluorescence of hyaluronidase preparations (at equal protein concentration) has a complex dependence on the degree of enzyme modification (Fig. 4A). It is seen that when the value of the latter is more than 60%, considerable conformation changes take place, which are caused by chemical modification of hyaluronidase. One may assume that as the result of conformation changes at modification degree of 70–90% (Fig. 4A) a sharp and noticeable decrease of heparin inhibition is attained (Fig. 3B). Data of spectrofluorometric study of heparin inhibition also confirm this. With that purpose, we determined dependence of intensity of intrinsic fluorescence of various hyaluronidase derivatives (the same preparations as mentioned above and at similar protein concentration) on the presence of increasing heparin concentrations (heparin titration). The resulting obtained curves of quenching of protein fluorescence by heparin were linearized in half-logarythmic plot (fluorescence intensity value depending on logarythm of heparin concentration) and tangent (according to the slope of initial section of this curve) was determined. The value of this tangent was proportional to observed changes occurring in enzyme structure in the presence of increasing heparin concentrations and registered by the intensity value of intrinsic fluorescence of protein derivatives. Determination of dependence of obtained tangent values (indicated as tgφ) on modification degree of hyaluronidase showed (Fig. 4B) that established dependence is rather similar to the earlier obtained one without heparin (Fig. 4A). Established similarity definitely indicates the dominating role of conformational changes brought about in hyaluronidase structure by modification (at modification degree more than 70%) in respect to decrease of heparin inhibition.

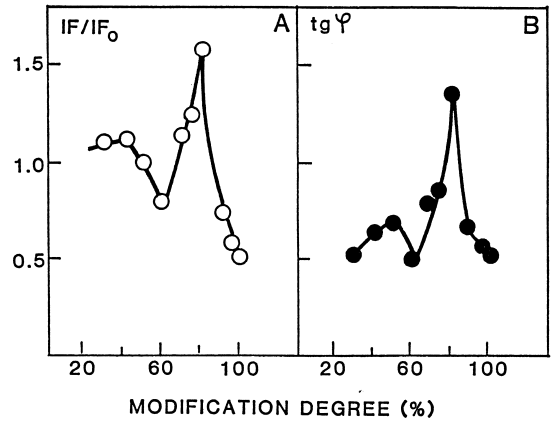

Fig. 4. Data of spectrofluorometric study of hyaluronidase derivatives (at similar concentration of protein in cuvette). (A) Effect of the degree of hyaluronidase modification on the value of relative intensity (IF/IF$_o$) of its intrinsic fluorescence, where fluorescence intensity is indicated as IF$_o$ for native and IF for corresponding hyaluronidase derivative; (B) value of changes of structural organization of hyaluronidase (tgφ) at interaction with increased heparin concentrations for enzyme derivatives with various modification degrees.

3.4. Importance of rather deep modification of hyaluronidase

The above mentioned statement about determining the impact of conformational changes of hyaluronidase induced by modification (at its respective depth) on the inhibition value of enzyme activity by heparin was emphasized by experimental data (not shown), which demonstrated that during denaturating electrophoresis (in the presence of sodium dodecyl sulfate), modified derivatives with small (20–40%) and considerable (60–100%) degree of modification did not differ by the value of their mobility. It means that there is no attachement of additional aldehyde dextran molecules to previous modified hyaluronidase. The increase of the modification degree is stipulated mainly by interaction of most veiled amino groups of the enzyme (which become available for this interaction due to the conformational changes) with polysaccharide attached already. These results, in conjunction with the above mentioned data show the special importance of modification of hard-to-reach amino groups of hyaluronidase for higher stabilization of its enzyme activity against heparin inhibition.

4. Conclusion

One may suggest that consecutive chemical modification of hyaluronidase by its surface amino groups will prove to be an effective approach for further study of heparin inhibition of this enzyme and also for the development of efficient therapeutic preparations of hyaluronidase, improving tissue flow and suitable for systemic administration into blood stream.

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