ANTICOAGULANT ACTIVITY OF HEPARIN: SEPARATION OF HIGH-ACTIVITY AND LOW-ACTIVITY HEPARIN SPECIES BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ANTITHROMBIN

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1. Introduction

Heparin is a glycosaminoglycan with unique biological properties, such as the ability to prevent blood from clotting. This anticoagulant activity is largely or wholly due to the interaction between plasma antithrombin (antithrombin III) and heparin, leading to an increased rate of inactivation of a number of the enzymes involved in the coagulation mechanism [1,2]. It has not been possible to define the structural properties responsible for the antithrombin-activating effect of heparin, although certain features such as a minimal mol. wt. and a high degree of *N*-sulfation appear to be essential [3,4].

In the present study heparin was separated by affinity chromatography on antithrombin-substituted Sepharose into two distinct fractions, one with high affinity and one with little or no affinity for the protein. The anticoagulant activities of the two fractions differed greatly, the high-affinity fraction having about 300 B.P. units/mg whereas the low-affinity fraction was almost inactive. A preliminary characterization of the two heparin fractions failed to reveal any structural dissimilarities, apart from a slight difference in charge density.

2. Materials and methods

Heparin (stage 14, isolated from pig intestinal mucosa), obtained from Inolex Pharmaceutical Div., Park Forest South, Ill., USA, was purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl, essentially as described elsewhere [5]. The purified material had an anticoagulant activity of 190 B.P. units/mg.

Radioactive heparin was isolated from mouse mastocytoma tissue, after labeling in vivo with inorganic [^{35}S]sulfate [6] or in vitro with [^{14}C]glucose [7].

Purified human thrombin with a stated activity of about 2500 NIH units/mg was kindly given by Dr M. Miller-Andersson, AB Kabi, Stockholm.

Human and bovine antithrombin were isolated from plasma by affinity chromatography on heparin-Sepharose and further purified by ion exchange chromatography and gel chromatography [8]. Bovine antithrombin was coupled to Sepharose and used as affinity matrix, as described below; human antithrombin was used only in the heparin activity assays.

Purified bovine antithrombin was coupled via amino groups to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in the presence of excess amounts of heparin. The heparin (previously treated with acetic anhydride [9] in order to acetylate any free amino groups present) was added as an attempt to shield the heparin-binding site of the antithrombin molecule from binding to the Sepharose beads. Activated Sepharose (5 ml) was reacted at pH 8.0 [10] with 50 mg of antithrombin in the presence of 300 mg of heparin. Amino acid analysis of the antithrombin-substituted Sepharose indicated a protein content of about 7 mg/ml of final gel suspension.

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Methods for the determination of uronic acid and radioactivity have been described [11]. The dry-weight of heparin samples was determined after drying over magnesium perchlorate in vacuo to constant weight. Duplicate samples invariably differed by less than 5%.

Anticoagulant activities were determined by the British Pharmacopoeia (B.P.) whole-blood assay [12]. Alternatively, the antithrombin-activating effect of heparin was measured by a procedure, based on the heparin cofactor activity method developed by Ødegård et al. [13]; the procedure is outlined in the legend to fig.2, and will be described in detail elsewhere.

Affinity chromatography of heparin on immobilized antithrombin was carried out as follows. Samples of heparin in 1 ml of 0.2 M NaCl–0.1 M Tris-HCl, pH 7.4, were applied to a column containing 3 ml of antithrombin-Sepharose gel, equilibrated with the same buffer at 4°C. The column was washed with buffer until the effluent was free from uronic acid or radioactivity, and was then eluted with a linear salt gradient (for further details, see the legend to fig.1). Effluent fractions were analyzed for uronic acid by the carbazole reaction and/or for radioactivity. Appropriate fractions were pooled, dialyzed against water and lyophilized. Samples were precipitated with cetylpyridinium chloride from 1 M NaCl and recovered as sodium salts [5] before determination of anticoagulant activity.

3. Results and discussion

3.1. *Affinity chromatography of heparin on antithrombin-Sepharose*

The fractionation of 1 mg of heparin by affinity chromatography on antithrombin-Sepharose is illustrated in fig.1. A portion of the material appeared in the breakthrough-fraction (0.2 M NaCl wash) while another portion emerged during gradient elution. The latter component comprised 38% of the uronic acid-containing material recovered from the gel; with ^{35}S- or ^{14}C-labeled heparin the corresponding values were 64% and 50%, respectively. The total recovery of radioactivity and uronic acid was invariably better than 90% of the material applied to the column.

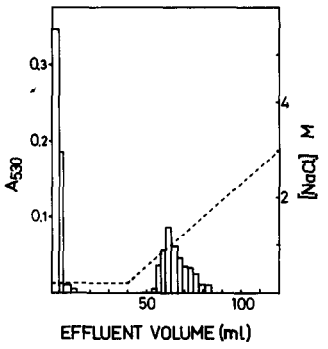

Fig.1. Chromatography of heparin on antithrombin-Sepharose. A sample (1 mg) of heparin, dissolved in 1 ml of 0.2 M NaCl–0.1 M Tris-HCl, pH 7.4, was applied to a 3 ml-column of antithrombin-Sepharose, equilibrated with the same buffer at 4°C. After washing with 40 ml of buffered 0.2 M NaCl, the column was eluted with sodium chloride (linear gradient from 0.2 M to 3 M concentration) in 0.1 M Tris-HCl, pH 7.4. Effluent fractions were analyzed for uronic acid by the carbazole reaction (A_{530}), (- - - - -) NaCl concentration.

Re-chromatography of once adsorbed, eluted and dialyzed heparin resulted in quantitative retention of the material by the gel. Conversely, the breakthrough fraction did not yield any adsorbed components on re-application to the gel. These results show the occurrence of at least two types of heparin, differing markedly in their affinity for antithrombin.

In preparative experiments, the antithrombin-Sepharose column was deliberately overloaded by applying 8 mg portions of heparin to 3 ml of gel. After extensive washing with buffered 0.2 M NaCl, the adsorbed polysaccharide was eluted in a single step with buffered 3 M NaCl. When the breakthrough fraction was reapplied to the antithrombin-Sepharose column (re-equilibrated with buffered 0.2 M NaCl) another portion of material was retained by the gel and could be eluted by salt. Three such successive passages through the gel, removing progressively smaller amounts of material, were required to virtually deplete the heparin solution of components having affinity for the column under the conditions used. The analyses which follow refer either to material eluted with salt after an initial passage of heparin solution through the antithrombin-Sepharose column (high-affinity heparin), or to heparin depleted of such components by three

Table 1
Anticoagulant activity (B.P. method) and uronic acid content of heparin, before and after fractionation on antithrombin-Sepharose

Preparation	Anticoagulant activity Recovery[a]	(B.P. units/mg dry weight)	Uronic acid content (% of dry weight)
Starting material	100	190	29.2
Low-affinity fraction	7	19	24.6
High-affinity fraction	54	285	29.9

[a] Per cent of starting material.

successive passages through the column (low-affinity heparin).

4.2. *Anticoagulant activity of high- and low-affinity heparin*

The anticoagulant activities (B.P. method) of heparin, measured before and after fractionation on the antithrombin-Sepharose gel, are shown in table 1. High-affinity heparin displayed exceptionally high activity; in contrast, low-affinity heparin was almost inactive. Essentially similar relations were found by the antithrombin-activation assay (fig.2); however, low-affinity heparin showed higher activity relative to the starting material than in the B.P. procedure.

In spite of almost complete recovery of uronic acid, the high-affinity and low-affinity heparin fractions only accounted for approx. 60% of the anticoagulant activity (B.P. units) of the starting material. The reason for this discrepancy is unknown. Recombination of the two fractions did not increase the activity beyond the calculated sum.

4.3. *Structures of high- and low-affinity heparins*

The ratio of *N*-sulfate to total sulfate was determined with [^{35}S]heparin, by paper electrophoretic separation of deamination products [14]; the values obtained were 0.36 and 0.32 for high- and low-affinity heparin, respectively. Further, the two heparin fractions displayed similar distribution of *N*-sulfate groups, as judged from the elution profiles obtained on gel chromatography [11] of deamination products. Paper electrophoresis (pH 1.7; ref. [11]) of deamination products derived from high- and low-affinity [^{35}S]heparin revealed approx. similar relative amounts of mono- and di-*O*-sulfated disaccharide units. Finally, the uronic acid composition of [^{14}C]heparin was determined [15]; the ratio of [^{14}C]iduronic acid to total [^{14}C]uronic acid was 0.49 and 0.58 for high- and low-affinity heparin, respectively.

These results indicate that both the high- and the low-affinity materials conform to the current structural concept of heparin; however, they do not

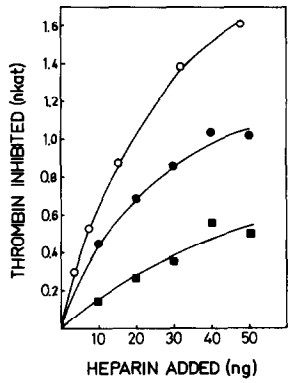

Fig.2. Antithrombin-activating properties of heparin, before and after chromatography on antithrombin-Sepharose. (•) Starting material; (○) high-affinity heparin; (▪) low-affinity heparin. Samples (100 μl) of antithrombin solution (50 μl/ml) and of heparin solutions of different concentrations were mixed and incubated for 5 min at 37°C. Thrombin (50 μl of a solution containing 90 NIH units/ml) was then added, and after additional incubation for 20 sec the remaining thrombin activity was measured by recording the release of *p*-nitroaniline (absorbing at 405 nm) from the synthetic thrombin substrate, Bz-Phe–Val–Arg-pNS (S-2160; AB Kabi, Sweden). The results are expressed as nkat thrombin inhibited; under the conditions of the assay this parameter is a function of the heparin activity.

reveal the cause of the difference in antithrombin affinity and anticoagulant activity. Gel chromatography on Sephadex G-100 also failed to distinguish between the two preparations, showing essentially similar molecular-size distributions. In contrast, on ion-exchange chromatography (DEAE-cellulose; see [11]) the low-affinity heparin emerged slightly but distinctly ahead of the high-affinity heparin, suggesting a somewhat higher net negative charge density in the latter material. The significance of this finding is unknown.

After conclusion of the present study a report appeared, showing the fractionation of heparin into two distinct forms by sucrose density gradient centrifugation in the presence of antithrombin [16]. The two fractions appear similar to the heparin fractions obtained by affinity chromatography; apparently, the separation of high-activity heparin from low-activity heparin may be accomplished by either procedure. The antithrombin-Sepharose method is the more suitable one for large-scale separations; in recent experiments the procedure has been successfully scaled up to achieve fractionation of 50 mg of heparin in a single run.

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