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Purification of the monoclonal heparin antibody H-1.18

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Abstract

An antibody of the (immunoglobulin) IgG_1 subclass against heparin was purified. Here we report on the purification of the heparin antibody. Ammonium sulfate precipitation was performed and showed a high purity of the precipitate. In the heparin radioimmunoassay it showed a high heparin binding. Capillary electrophoresis showed that albumin and other proteins were separated from the heparin antibody. The purification method allowed a large scale production of the heparin antibody.

Keywords: Monoclonal antibodies; Heparin; Saccharides; Polysaccharides

1. Introduction

Heparin belongs to the glycosaminoglycans and is a negatively charged heterogeneous polysaccharide. It consists of α-1→4 linked derivatives of the disaccharide unit 2-amino-2-deoxy-D-glucopyranose. The most frequent glucosamine unit is N-sulfated ($GlcNSO_3$), although occasionally it is N-acetylated (GlcNAc) [1].

Heparin is widely used as an antithrombotic and anticoagulant agent in clinical use. It functions as an anticoagulant drug by binding antithrombin III and accelerating the rate at which the protein complex inactivates the serine proteases of the haemostatic system [1,2].

Glycosaminoglycans are weak antigens. These compounds have to be bound to a carrier to produce antibodies. Monoclonal antibodies were produced against chondroitin [3], keratan sulfate [4], heparan sulfate [5] and heparin [6].

A polyclonal antibody against heparin was described. It bound to heparin, which was linked to a polymer consisting of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide [6]. An antibody of IgM (immunoglobulin M) class against heparin was produced. The antibody showed an improved binding of heparin in the presence of a polylysine surface [7].

H-1.18 is a murine monoclonal antibody (Mab) of the IgG_1 isotype which is directed against heparin [8]. The antibody is produced by hydridoma cells. The cell culture supernatant contains foetal bovine serum. For the purification of the antibody, methods are used which remove other proteins from the antibody.

Purification methods of monoclonal antibodies include precipitation, chromatographic and electrophoretic methods. The most frequently used purification of monoclonal antibodies is ammonium sulfate precipitation [9].

Chromatographic purifications are frequently performed by protein A [10] and protein A/G chromatography [11]. For the analysis of the antibody, electrophoretic methods such as PAGE [12] or capillary electrophoresis are used. Capillary electrophoresis methods separate proteins according to their relative molecular mass [13]. Methods for immunoassays in bare silica capillaries are in development [14].

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Here we report on different methods for the purification of the heparin antibody. The heparin binding was analyzed by a heparin radioimmunoassay. Capillary electrophoresis demonstrates the purity of the heparin antibody.

2. Experimental section

2.1. Materials

Cell culture experiments were performed with 1640 RPMI medium, L-glutamine and non-essential amino acids from Gibco Laboratories (Paisley, Scotland). Foetal calf serum was obtained from Boehringer (Mannheim, Germany). HAT medium supplement and the penicillin–streptomycin supplement were from Sigma (St. Louis, USA).

Ammonium sulfate and sodium chloride were obtained from Serva (Heidelberg, Germany). Radiolabeled low-molecular-mass heparin (LMMH)–tyramine–^{125}iodine, donkey-anti-mouse serum and normal mouse serum were prepared from Immunodiagnostics (Bensheim, Germany).

Unfractionated heparin was from Braun (Melsungen, Germany). Bovine serum albumin (BSA), mouse immunoglobulin, Tween 80 monoelate polyethylene glycol and glycerol were obtained from Sigma (Deisenhofen, Germany), immunoglobulin (Sandoglobulin) was bought from Sandoz (Nürnberg, Germany). Protein A Sepharose and protein A/G which was cross-linked to agarose was obtained from Pierce (Beijerland, Netherlands).

Diaflo-ultrafiltration membranes (cut off M_r $100 \cdot 10^3$) from Amicon (Beverly, USA) were used for dialysis. The cell culture experiments were performed using a laminar flow box from Heraeus (Hanau, Germany). The fast protein liquid chromatography system was from Pharmacia (Freiburg Germany).

Capillary electrophoresis was performed by a PACE 2050 instrument from Beckmann (Fullerton, CA, USA). A 50 cm long fused-silica capillary (75 μm I.D.) from the same supplier was used.

2.2. Hybridoma cell culture supernatant

Hydridoma cells producing the heparin antibody H-1.18 were grown in 1640 RPMI medium containing 5% foetal calf serum. The culture supernatants were harvested by centrifugation (250 g) and stored at −40°C.

2.3. Protein A/G affinity chromatography

The protein A/G affinity chromatography was performed using a fast flow liquid chromatography system. A column (3 cm×0.5 cm I.D.) was packed with 3.0 ml of protein A/G. Cell culture supernatant (40.0 ml) was introduced to the column by an automatic sample loop (50.0 ml). The binding buffer was 0.01 M Tris·HCl (pH 7.0). The elution buffer consisted of 0.01 M glycine·HCl (pH 3.0). Fractions of 2.0 ml were collected from the IgG peak. After elution, the fractions were neutralized immediately by 1.0 M phosphate-buffered saline solution (pH 7.2).

2.4. Protein A and protein A/G purification

Cell culture supernatant (10.0 ml) was incubated with 3.0 ml of protein A or protein A/G respectively. A 10.0 ml volume of 0.067 M phosphate buffer (pH 7.0) containing 1% Tween 80 was added. Incubation of the protein A or protein A/G gel was performed for 18 h while mixing at room temperature. The gel was mildly centrifuged (2000 g, 3 min). The sediment was washed twice with 0.9% saline solution. The elution was performed 4 times using 10.0 ml 0.1 M glycine·HCl buffer (pH 3.0). After centrifugation the IgG fractions were immediately neutralized by 1.0 M phosphate buffer (pH 7.2).

2.5. Ammonium sulfate precipitation

Ammonium sulfate precipitation of the cell culture supernatant was carried out according to [15]. It was performed with 20, 30, 40 and 50% (w/v) solutions. The precipitate was dialyzed against water (pH 7.0) containing 1% Tween 80 for stabilization.

The antibody production was scaled up as follows: the cell culture supernatant (400 ml) was precipitated by a 30% ammonium sulfate solution. The precipitate was redissolved in 20.0 ml distilled water containing 1% Tween 80 and dialyzed for 12 h (pH 7.0). The purified antibody was concentrated and stored at −20°C.

2.6. Competitive radioimmunoassay of heparin

Heparin and LMMH (10 to 10^{-7} mg/ml) were incubated with ^{125}iodine labeled LMMH–tyramine and 10.0 μl purified heparin antibody. Heparin binding was detected by immunoprecipitation with a donkey-anti-mouse Fab-specific antibody. Centrifugation was performed at 2000 *g*. The binding of the antibody was measured by the γ emission of ^{125}iodine labeled LMMH–tyramine [20].

2.7. Capillary electrophoresis of the heparin antibody

Capillary electrophoresis was performed by a PACE 2050 instrument from Beckmann. A fused-silica capillary (50 cm×75 μm I.D.) from the same supplier was used. Capillary electrophoresis was performed using a borate buffer containing 0.3% sodium tetraborate and 0.4% boric acid (pH 9.0). High pressure injection was used for 30 s. High voltage (30 kV, 500 V/cm) was used. The temperature was 20°C and detection was performed at 200 nm. The data rate was set at 2 Hz and the range of absorbance was 0.2 au.. After each run the capillary was rinsed for 2.0 min by 0.01 *M* hydrochloric acid and 2.0 min by 0.01 *M* sodium hydroxide.

Purified heparin antibody (0.4 mg/ml) and different concentrations of heparin (1.2 to 833 n*M*) were coinjected for 1 s with high pressure injection. The incubation time was 30s. Capillary electrophoresis was performed as described before.

2.8. Protein test

The protein concentration of the purified antibody fractions was determined using the Lowry Protein Test.

3. Results

The heparin antibody H-1.1.8 was expressed in mouse hydridoma cells. They were fused from mouse liver cells and malignoma cells. The antibody is directed against heparin [6]. It is purified by chromatography and ammonium sulfate precipitation. The binding of heparin is characterized by a heparin radioimmunoassay and capillary electrophoresis.

3.1. Affinity chromatography using protein A and protein A/G

Protein A/G is a fusion protein which binds to all IgG subclasses of immunoglobulins. Fig. 1 shows the purification of 40.0 ml cell culture supernatant. The antibody was bound from 5.0 to 80.0 min onto the column. From 80.0 to 94.0 min a washing step was performed. It eluted albumin and other proteins from the column. The antibody was eluted by glycine·HCl

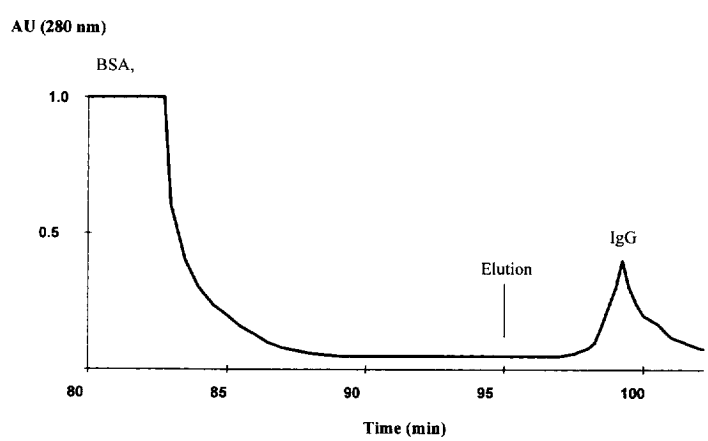

Fig. 1. The protein A/G fast liquid chromatography is shown. The first peak displays bovine serum albumin and the second peak the heparin antibody. The binding buffer was 0.01 *M* Tris (pH 7.0). The flow-rate was 0.1 ml/min. Elution was performed with a glycine·HCl buffer (pH 3.0), with a flow-rate of 2.0 ml/min.

(pH 3.0) from 95.0 to 110.0 min (Fig. 1). Fractions of 2.0 ml were collected. The antibody fractions contained immunoglobulin with a low heparin binding in the radioimmunoassay.

3.2. Protein A and protein A/G purification

A longer incubation time of the heparin antibody increased the heparin binding using protein A/G. A 10.0 ml volume of culture supernatant was incubated for 14 h with 3.0 ml protein A/G gel. The antibody had a 300% higher heparin binding and its concentration was about 100% higher than using fast flow liquid chromatography.

Protein A affinity chromatography was used to separate IgM and IgG from other proteins. The antibody obtained by protein A chromatography showed similar results to the protein A/G chromatography (Table 1).

Using a longer incubation time, the concentration of antibody was higher (226.4 μg/ml) (Table 1). The concentration of the antibody which was bound to protein A and protein A/G was similar. It showed a high heparin binding (9676.6 cpm) in the heparin radioimmunoassay.

3.3. Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed with 20, 30, 40 and 50% (w/v) aqueous solutions (Table 2). The protein concentration of the precipitate was lower for 20% (320 μg/ml) and 30% (268 μg/ml) precipitations than for the 40% (734 μg/ml) and the 50% (1423 μg/ml) ammonium sulfate precipitations. The highest activity of radioactive heparin–tyramine (19 374 cpm) was obtained by 30% ammonium sulfate precipitation.

Table 1
Activity and protein concentration of the heparin antibody H-1.18 with different purifications. The properties of heparin antibody purified by chromatographic and precipitation are compared

Purification	Bound LMMH–tyramine–^{125}I (cpm)	Protein (μg/ml)
Protein A/Gchromatography	–	200.4
Protein A/G purification	–	218.6
Protein A purification	9 676.6	226.4
Ammonium sulfate precipitation	12 155.0	2063.8

Table 2
Properties of different ammonium sulfate precipitates of the heparin antibody. Cell culture supernatant (50.0 ml) was precipitated by 20, 30, 40 and 50% (w/v) ammonium sulfate. The heparin binding was determined by radioimmunoassay

Ammonium sulfate (%, w/v)	Bound LMMH–tyramine–^{125}I (cpm)	Protein (μg/ml)	Ratio activity/protein (cpm ml/μg)
20	17 198	320	53.7
30	19 374	268	72.3
40	9 257	734	12.6
50	4 885	1423	3.4

Table 2 shows the ratio between the bound radioactive heparin (cpm/min) and the protein. The highest ratio was obtained for the 30% ammonium precipitate. The data indicate that at 20% ammonium sulfate, precipitation is not complete and that at 40% and 50% ammonium sulfate, precipitates contain large amounts of other proteins, (i.e. albumin). Thus the precipitation was performed using a 30% (w/v) ammonium sulfate.

Excessive ammonium sulfate and proteins ($M_r < 100 \cdot 10^3$) were removed by dialysis. The precipitation of the antibody during dialysis was inhibited by addition of 1% Tween 80.

The purified antibody showed a high heparin binding (12.155 cpm) and a 10-fold higher protein concentration than the antibody purified by chromatographic methods (Table 1).

3.4. Analysis of the heparin antibody

Fifteen lots of the heparin antibody were produced by 30% ammonium sulfate precipitation and dialysis as described before. The binding of the heparin antibody was determined by the γ emission of ^{125}iodine-labeled LMMH–tyramine (Fig. 2). The activity was 11 110.6±3420.7 cpm ($n=15$, $x=$ mean±S.D.).

The purification of the antibody was analyzed using capillary electrophoresis. The antibody was determined in cell culture supernatant and ammonium sulfate precipitates. It was detected at 8.3±0.5 min and albumin at 10.5±1.1 min ($n=3$, $x=$

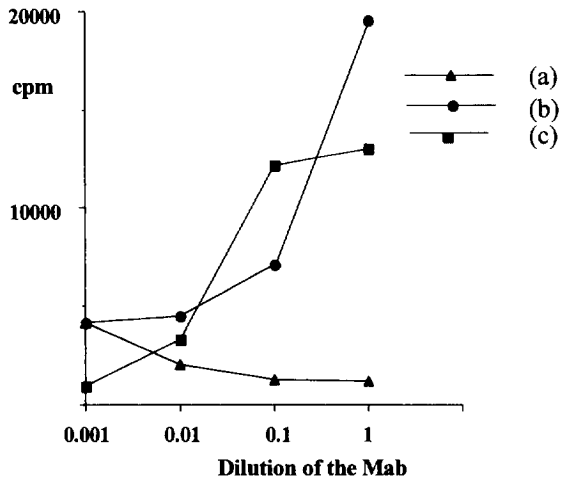

Fig. 2. The binding of the antibody of heparin was measured by a heparin radioimmunoassay. The dilution of the Mab is displayed on the x-axis. The counts per minute are displayed on the y-axis. (a) shows cell culture supernatant containing heparin antibody, (b) displays the 30% ammonium sulfate precipitate and (c) displays the purifed antibody.

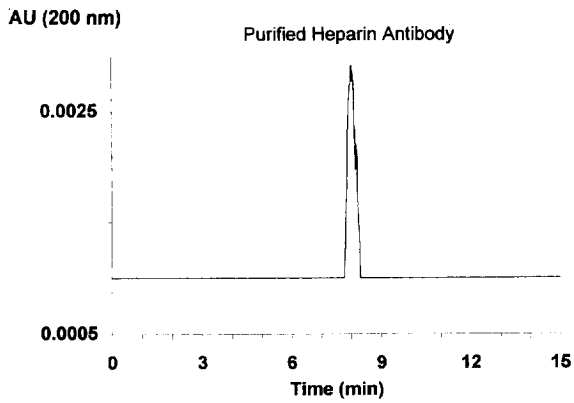

Fig. 4. The electropherogram of the purified heparin antibody is displayed. The purification was performed by 30% ammonium sulfate precipitation followed by dialysis, ($M_r > 100 \cdot 10^3$).

mean±S.D.) (Fig. 3). The electropherogram of the purified heparin antibody shows that other proteins were not present (Fig. 4).

The complex formation of heparin and the heparin antibody was analyzed using capillary electrophoresis. The heparin antibody and heparin with different concentrations of heparin (between 1.2 and 833 nM were coinjected. With increasing amounts of heparin the migration time of the heparin antibody decreased (Fig. 5). The peak area of the heparin antibody decreased with increasing amounts of heparin. Plotting the logarithm of the peak area versus the logarithm of the heparin, a logarithmic equation ($y = -0.14 \ln x + 1.16$) was obtained. The correlation coefficient was 0.96.

Purifications like protein A and protein A/G affinity chromatography resulted in a low heparin binding. The ammonium sulfate precipitation and

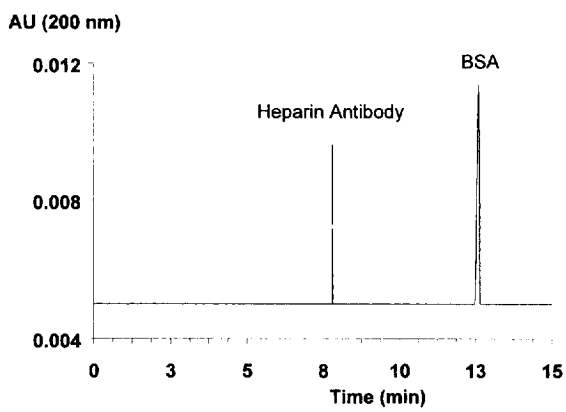

Fig. 3. High-performance capillary electrophoresis was performed to determine the purification of the antibody using a borate buffer system (pH 9.0). Display of different migration times of immunoglobulin and albumin in the cell culture supernatant.

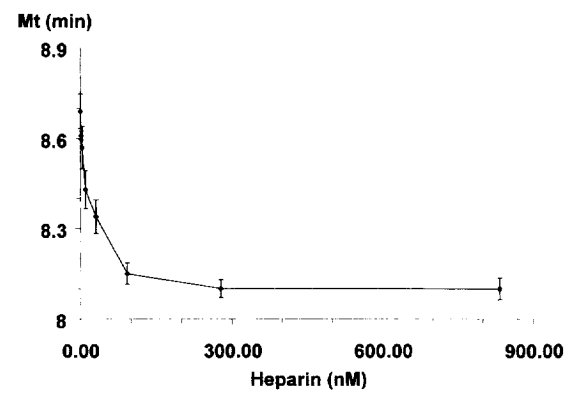

Fig. 5. The capillary electrophoresis of the heparin antibody after addition of different concentrations of heparin is demonstrated. Adding heparin to the heparin antibody the migration time decreased. (n=3, x=mean±SD).

dialysis showed a high heparin binding. The heparin binding was tested using a radioimmunoassay for heparin and capillary electrophoresis.

4. Discussion

The purification of the heparin antibody was evaluated using a combination of chromatographic, precipitation and electrophoretic methods.

Protein A/G chromatography is widely used for the purification of mouse monoclonal antibodies from IgG subclasses without interference from IgA, IgM and murine serum albumin [16]. It is a genetically modified protein and combines the IgG binding profiles of protein A and protein G. Protein A/G contains four Fc binding domains from protein A and two Fc domains from protein G.

Protein A/G fast flow liquid chromatography was used to separate the heparin antibody from albumin and other proteins which are present in the foetal calf serum and cell culture supernatant. Low pH is required for the elution. Immediate neutralization of the eluted antibody was performed.

Protein A is immobilized on cross-linked agarose matrix and allows the isolation of mouse IgG_1. This matrix was used to bind the heparin antibody because it is of mouse origin.

Differences between the results of fast liquid chromatography and incubation with protein A and protein A/G material show that the specific binding of the antibody to the column material needs a longer incubation.

Precipitation methods include caprylic acid [17], euglobulin [18] and ammonium sulfate precipitation [15]. The precipitation was performed with 20, 30, 40 and 50% (w/v) ammonium sulfate solutions. The precipitate of the 30% ammonium sulfate precipitation showed high amounts of immunoglobulins and low amounts of albumin.

The precipitate was redissolved in water containing 1.0% Tween 80 for stabilization according to [19]. Dialysis was performed with an ultrafiltration membrane to remove excessive ammonium sulfate and proteins.

A radioimmunoassay was used to measure the concentration of heparin. As tracer, radiolabeled LMMH–tyramine was used [21]. It was labeled by ^{125}iodine using chloramine T oxidation. The labeled (LMMH)–tyramine–iodine was purified from free ^{125}iodine by Sephadex G-25 gel permeation chromatography.

The binding of heparin and low-molecular-mass heparins to the antibody was measured using a heparin radioimmunoassay [8]. In short: heparin is incubated with LMMH–tyramine–iodine and the heparin antibody. Increasing amounts of heparin decrease the binding of LMMH–tyramine–iodine to the antibody. The precipitation of the heparin antibody is performed using a donkey-anti-mouse Fab-specific antibody. The γ emission is measured. The inhibition of the LMMH–tyramine–iodine is calculated in percent. Sulfated monosaccharides, oligosaccharides and low-molecular-mass heparins show a different affinity to the heparin antibody [8].

Several capillary electrophoresis methods were developed for the detection of serum proteins [22]. Capillary electrophoresis of the heparin antibody was performed. The method, which was used separated proteins, i.e., albumin from immunoglobulins.

The interaction of the heparin antibody and heparin was studied using capillary electrophoresis. The heparin antibody was incubated with heparin before the electrophoresis was performed. As a consequence of the interaction the migration time decreased. The peak area of the heparin antibody decreased with increasing concentration of heparin. Thus capillary electrophoresis can be used to characterize the binding of the heparin antibody to heparin.

The experiments show that the heparin antibody has been purified. It was characterized by chromatographic immunological and electrophoretic methods.

5. Abbreviations

AU	= absorption
BSA	= bovine serum albumin
cpm	= counts per minute
HCl	= hydrochloric acid
LMMH	= low-molecular-mass heparin
LMMH–tyramine	= low-molecular-mass heparin–tyramine
Mab	= monoclonal antibody

M_r = relative molecular mass
rpm = rounds per minute

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