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IMMUNO-AFFINITY PURIFICATION OF HEPARINASE

ROBERT J. LINHARDT*, ZOHAR M. MERCHANT and DAVID W. PERSINGER
Division of Medicinal Chemistry and Natural Products, College of Pharmacy, University of Iowa, Iowa City, IA 52242, U.S.A. [*Tel.* (319) 353–3955]

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Abstract—1. Polyclonal IgG rabbit antibodies were prepared against a purified heparinase from *Flavobacterium heparinum*.

2. Immuno-affinity purification of crude and partially purified heparinase is described.

3. The resulting enzyme was of comparable purity to that prepared using the standard multistep purification scheme.

4. The antibodies prepared were found to increase the activity of bound heparinase.

INTRODUCTION

Heparinase, heparin lyase EC 4.2.2.7, acts at the major glycosidic linkage occurring in heparin. The importance of heparin as an anticoagulant has heightened interest in the preparation and purification of heparinase. Heparinase has been used to study heparin's structure (Merchant *et al.*, 1985), anticoagulant activity (Linhardt *et al.*, 1982) and to establish its presence in complex proteoglycans (Kanwar and Farquhar, 1979). Heparinase has been applied to the preparation of low molecular weight heparins with anticoagulant (Linhardt *et al.*, 1982), antitumor (Folkman *et al.*, 1983) and complement activation inhibition activities (Sharath *et al.*, 1985). Immobilized heparinase has been used to deheparinize extracorporeal circuits (Langer *et al.*, 1982).

Antibodies prepared against a partially purified heparinase preparation were shown to have no effect on its enzymatic activity (Klein *et al.*, 1983). This observation raised the question of whether the antibodies were directed against heparinase itself or some contaminating protein (Klein *et al.*, 1983). Difficulties have been reported in the preparation of antibodies directed against hyaluronate lyase (Ingham *et al.*, 1984), an enzyme closely related to heparinase.

We report the preparation of antibodies directed against heparinase and their use in the immuno-affinity purification of heparinase.

MATERIALS AND METHODS

Materials

Immuno-diffusion plates were obtained from Hyland Diagnostics (Deerfield, Ill., U.S.A.). The Cappel Pro-Blot kit, was from Cooper Biomedical (Melvern, Pa, U.S.A.). Protein-A-Sepharose and cyanogen bromide-activated-Sepharose were from Pharmacia (Piscataway, N.J., U.S.A.). Complete and incomplete Freunds Adjuvant were obtained from Difco (Milwaukee, Wis., U.S.A.). All other materials were obtained as previously reported (Merchant *et al.*, 1985).

*To whom all correspondence should be addressed.

Assays

Heparinase activity was assayed either by following the absorbance of the products at 232 nm or in crude preparations by measuring substrate disappearance using Azure dye (Linhardt *et al.*, 1984). Protein concentration was determined using Bradford reagent (Bradford, 1976). Antibody titer was measured on Ouchterlony precipitin plates (Ouchterlony and Nilsson, 1978) and on nitrocellulose using an antirabbit IgG peroxidase conjugate (Gershoni and Palade, 1983). Ouchterlony assays were performed at serum dilutions from 1- to 16-fold using from 0.5 to 5 mg hydroxylapatite purified heparinase as antigen. Antibody titer was estimated on nitrocellulose using 1 μg of chromatofocused or purified heparinase as antigen at dilutions of 10- to 10,000-fold. Assays were performed on 1 ml of purified antibody preparation or serum at dilutions of 15- to 15,000-fold.

Electrophoresis

SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was run in tube gels and IEF–(isoelectric focusing) PAGE on a horizontal flat-bed (Linhardt *et al.*, 1984), both were stained with Coomassie Blue (Maizel, 1971).

Preparation of heparinase for antibody production

Heparinase was prepared fermentatively (Galliher *et al.*, 1981) and purified by batch hydroxylapatite chromatography, batch QAE Sephadex chromatography, chromatofocusing and gel-permeation chromatography (Yang *et al.*, 1985).

Preparation of antibodies

A female new Zealand white rabbit 2–3 kg was used to prepare antibodies. Two volumes complete (immunization) or incomplete (boosters) Freunds adjuvant was combined with 1 vol heparinase in PBS (phosphate buffered saline) (0.9% NaCl and 150 mM sodium phosphate, pH 7.2). The gel, containing heparinase, was finely ground with PBS, combined with adjuvant and emulsified. The injection (2–3 ml) was administered s.c. in the right and left flank (the immunization was also given in both right and left shoulders). The immunization was given on day 0 (150 μg denatured unstained heparinase) and the six boosters were given at 16-day intervals starting on day 16. The blood, drawn 6 days before immunization and 7 days after each booster, was held at 37°C for 30 min to clot. The clot was pulled away from the wall with a wooden applicator stick and the sample was stored overnight at 4°C. The serum was

decanted from the clot, centrifuged at 2500 rev/min for 15 min and stored at −70°C.

Purification of antibodies

Post-immune serum (12 ml) was precipitated with an equal volume of saturated ammonium sulfate at 20°C (McCans *et al.*, 1974). After stirring for 30 min and centrifuging at 10,000 *g* for 20 min the pellet, containing IgG, was resuspended in 4 ml of buffer (10 mM sodium phosphate and 150 mM sodium chloride, at pH 7), dialyzed against 1 l. of the same buffer and frozen for binding to CNBr–Sepharose.

Immobilization of antibodies

Purified antibody preparation (13 mg) was coupled to 6 ml of CNBr–Sepharose 4B (Linhardt *et al.*, 1984). The beads were blocked with 0.2 M glycine and washed with 20 bed vol of high (pH 8.5) and low (pH 4) buffers containing 0.5 M sodium chloride.

Affinity purification of heparinase

The IgG–Sepharose beads were packed into a 6 ml polyethylene column and equilibrated with 10 mM sodium phosphate buffer (pH 7). Excess enzyme in the same buffer was loaded onto the column and bound. The column was then washed of contaminating proteins with 1 bed vol of each of 0.1, 1 and 0.01 M sodium phosphate buffer (pH 7.4). Release of heparinase was accomplished by applying 1 M propionic acid (pH 2.5), collecting the acidic fractions and immediately neutralizing (to pH 7) with 1 M sodium carbonate. These fractions were dialyzed against 100 vol of 10 mM Tris–HCl buffer (pH 7). The column was regenerated by washing the column as described, following antibody immobilization and was stored in 10 mM sodium phosphate buffer (pH 7) containing 0.05% sodium azide. This column was used and stored at 4°C.

RESULTS AND DISCUSSION

Heparinase was prepared fermentatively from *Flavobacterium heparinum* (Galliher *et al.*, 1981). After cell disruption, nucleic acids were removed with protamine sulfate. This crude heparinase preparation was then purified (Table 1) by hydroxylapatite and QAE batch chromatography, followed by chromatofocusing and gel-permeation chromatography (Linhardt *et al.*, 1984; Yang *et al.*, 1985). The purified heparinase gave a major band (>90% by scanning densitometry) on SDS–PAGE (Fig. 1) and IEF–PAGE corresponding to the reported molecular weight and isoelectric point for this enzyme (Linhardt *et al.*, 1984; Yang *et al.*, 1985). Heparinase activity could be recovered from the unstained band on IEF–PAGE. The purified heparinase, as well as the cut-out of the SDS–PAGE band (both stained and unstained) comprised of denatured heparinase was used to prepare antibodies.

Antiheparinase in rabbit serum was measured by ELISA on nitrocellulose and titer was estimated by scanning densitometry using a series of dilutions. The antibody titer was 8–40 μg/ml despite the amount of antigen administered (20–100 μg), or its processing (i.e. immunization with natured, denatured, stained or unstained antigen). Attempts to measure serum antibody by immuno-diffusion were not successful.

Serum antibodies were partially purified to give a preparation containing approx. 50% IgG (McCans *et al.*, 1974) which was used both to study its interaction with heparinase and for immobilization to Sepharose 4B.

Studies were undertaken to establish that the rabbit antibody preparation was interacting with heparinase and not some other minor protein which might be contaminating our purified enzyme preparation. These studies (Table 2) showed a rate enhancement instead of the anticipated rate decrease in the presence of antibody. This antibody is neither a precipitating antibody, as shown by its failure to give a positive Ouchterlony assay, nor does it decrease the enzymatic activity of heparinase. A second experiment examined the binding of heparinase-antibody complex to Protein-A-Sepharose. This experiment (Table 2) clearly establishes that the IgG present in the purified antibody preparation is binding to heparinase.

Purified antibody preparation was coupled to 6 ml of Sepharose 4B and loaded into a column. The crude enzyme preparation (7.5 mg) or the hydroxylapatite heparinase (2.5 mg) was then applied to the column. Heparinase represents <0.1% of the protein present in these preparations. The column retained approx. 50% of the enzymatic activity. No heparinase eluted even after washing with 1 M sodium phosphate (pH 7) or with 5 M magnesium chloride in 100 mM Tris–HCl buffer (pH 7). Release of bound protein could be accomplished using 6 M ammonium thiocyananate, but activity could not be recovered even after dialysis. The protein eluting under these conditions gave a major band on SDS–PAGE at the same place as did the purified heparinase. Heparinase activity was released by washing the column with 1 M propionic acid (Table 1). Eluent with a pH of 2.5 was collected, neutralized immediately with 1 M sodium carbonate, and dialyzed overnight against 100 vol of 10 mM Tris–HCl buffer (pH 7). The immuno-affinity

Table 1. Purification of heparinase

Purification step	Protein (mg)	Sp. act. (U/mg)[a]	Tot. act. (U)
Protamine sulfate precipitated	340	0.1[b]	34[b]
Hydroxylapatite chromatography	93	0.3	31
QAE–Sephadex chromatography	28	0.9	25
Chromatofocusing	2.3	1.5	3.5
Gel-permeation chromatography	0.2	4.2	0.8

[a]Activity measured by increase in product absorbance at 232 nm, 1 U = 1 μmol of product formed/min (Linhardt *et al.*, 1984; Yang *et al.*, 1985).
[b]Activity measured by disappearance of substrate detected using Azure dye (Linhardt *et al.*, 1984; Yang *et al.*, 1985).

Table 2. Antibody–heparinase interaction

Sample[a]	Relative heparinase activity[b]
Heparinase + PBS (control)	1.0
Heparinase + Pre-immune serum (control)	0.8
Heparinase + Post-immune serum	1.6
Heparinase after Sepharose 4B (control)	1.0
Heparinase after Protein-A-Sepharose (control)	1.2
Heparinase + purified antibody preparation after Sepharose 4B	3.4
Heparinase + purified antibody preparation after Protein-A-Sepharose	0.0

[a]Hydroxylapatite purified heparinase (1 mg/ml protein) in PBS was used. Equal volumes (100 μl) of heparinase and diluted serum (5-fold in PBS) or purified antibody preparation were used.
[b]Protein-A-Sepharose or Sepharose 4B (0.5 ml) was packed in a column equilibrated with PBS through which the samples were passed and activity was measured in the effluent.

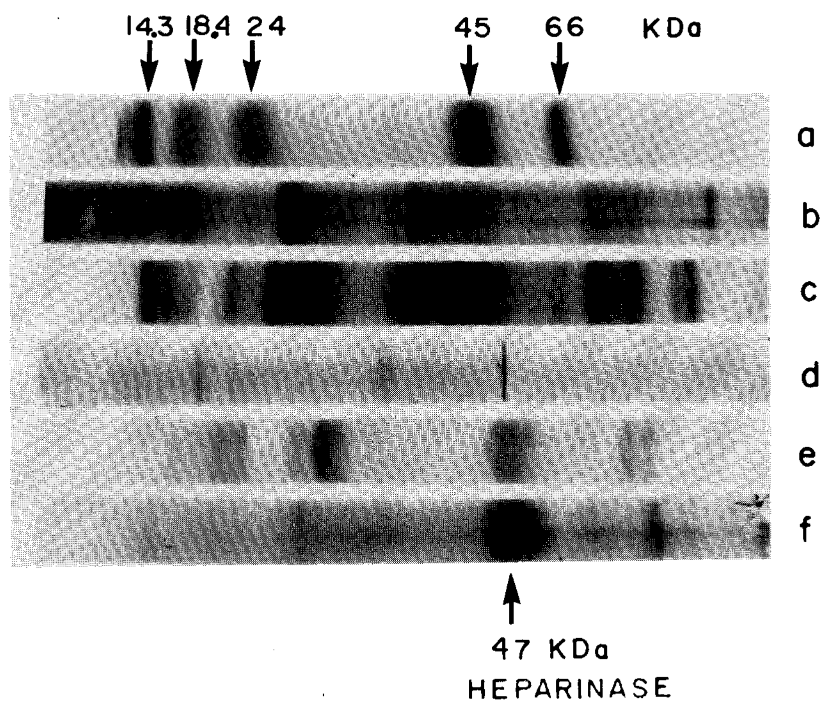

Fig. 1. SDS–PAGE: (a) 10 μg each of molecular weight markers; (b) 100 μg crude heparinase; (c) 100 μg hydroxylapatite heparinase; (d) 15 μg purified enzyme preparation; (e) 15 μg immuno-affinity purified heparinase prepared from crude heparinase; (f) 15 μg immuno-affinity purified heparinase prepared from hydroxylapatite heparinase.

purified heparinase (100 μg protein) had a specific activity of 0.5 U/mg. Exposure to propionic acid results in some catalytic inactivation of heparinase, however, the recovery of total activity is comparable to that observed in the multistep purification procedure.

In addition to being useful in affinity purification, this polyclonal antibody preparation will facilitate the screening of heparinase production by recombinant organisms.

SUMMARY

Polyclonal IgG rabbit antibodies were prepared against a purified Flavobacterial heparinase (>90% by SDS–PAGE). The antibodies were purified by ammonium sulfate precipitation (McCans *et al.*, 1974) yielding 8–40 μg/ml IgG in serum. The binding of heparinase-antibody complex to Protein-A-Sepharose and the rate enhancement of antibody bound heparinase establishes the interaction of this antibody to heparinase and not some minor contaminating protein. Immuno-affinity purification of heparinase was carried out and resulted in heparinase of comparable purity to that obtained using the standard multistep procedure.

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