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MONOCLONAL ANTIBODIES PREPARED AGAINST HEPARIN LYASE I AND THEIR REACTIVITY TOWARD HEPARIN LYASE I, II AND III

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Abstract—1. Six different monoclonal IgG mouse antibodies to heparin lyase I from *Flavobacterium heparinum* were prepared.

2. The monoclonal antibodies were used to detect heparin lyases I, II and III by dot-blotting immunoassay and by Western blotting.

3. Individual antibodies showed different reactivity toward the three heparin lyases.

4. The reactivity of two of the monoclonal antibodies was destroyed by exposing heparin lyases to sodium dodecyl sulfate.

5. The antibodies can be used to rapidly distinguish between the three heparin lyases.

INTRODUCTION

Heparin is a naturally occurring acidic polysaccharide that is most commonly known for it's capacity to augment the anticoagulant activity of antithrombin III. Heparin lyases are a general class of enzymes that are capable of specifically cleaving the major glycosidic linkages in heparin and heparan sulfate. Three heparin lyases have been isolated from *Flavobacterium heparinum* and purified to homogeneity (Lohse, 1992; Lohse and Linhardt, 1992). These lyases are designated as heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparinase II, no EC number) and heparin lyase III (heparitinase EC 4.2.2.8). The three purified heparin lyases differ in their capacity to cleave heparin and heparan sulfate: heparin lyase I primarily cleaves heparin, heparin lyase III specifically cleaves heparan sulfate and heparin lyase II acts equally on both heparin and heparan sulfate (Linhardt *et al.*, 1986; Linhardt *et al.*, 1990a; Lohse, 1992).

Heparin lyases have been used to establish the presence of heparin in mixtures of proteoglycans (Kanwar and Farquhar, 1979), to depolymerize heparin and heparan sulfate, to characterize the structure of the resulting oligosaccharides (Linhardt *et al.*, 1990a; Linhardt *et al.*, 1988; Merchant *et al.*, 1985; Turnbull *et al.*, 1988), to produce low molecular weight heparin preparations with anticoagulant and complement inhibitory activities (Linhardt *et al.*, 1982; Linhardt *et al.*, 1990b; Sharath *et al.*, 1985) and to remove heparin from the circulation (Langer *et al.*, 1982). However, studies utilizing heparin lyases are hampered by difficulties in purifying the enzymes from *Flavobacterium heparinum*, especially with regard to separation of the three enzymes from each other (Linhardt *et al.*, 1985; Lohse, 1992; Lohse and Linhardt, 1992). Specifically, the capacity of heparin lyase II to cleave both heparin and heparan sulfate makes it difficult to distinguish from heparin lyase I which cleaves heparin and heparin lyase III which cleaves heparan sulfate.

Previously, we described the production of polyclonal antibodies to heparin lyase I that were used to immunoaffinity purify heparinase from Flavobacterial cell homogenate (Linhardt *et al.*, 1985). The immunoaffinity purified enzymes had purity comparable to that of enzymes isolated by the conventional multi-step process, but elution of the heparin lyase from the immunoaffinity column required harsh conditions that greatly reduced enzyme activity (Linhardt *et al.*, 1985).

In the present study, we injected heparin lyase I into mice and used their B lymphocytes to form monoclonal antibody-producing hybridomas. The specificity of the monoclonal antibodies (MAbs) for each of the three heparin lyases was examined.

MATERIALS AND METHODS

Preparation of heparin lyases for antibody production

Heparin lyases I, II and III were isolated from *Flavobacterium heparinum* and purified to homogeneity as described previously (Lohse, 1992; Lohse *et al.*, 1992). Heparin lyase concentrations were determined using a Bio-Rad Protein Assay Kit (Richmond, CA, U.S.A.).

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Preparation of monoclonal antibodies

Six MAbs were prepared as described previously (Lohse, 1992). Briefly, purified heparin lyase I was injected into mice three times over a period of 70 days. The mouse spleens were harvested and lymphocytes were isolated from the splenocyte mixture. The lymphocytes were fused with mouse myeloma cells to produce hybridomas. The hybridomas were cultured and screened for production of antibodies to heparin lyase I. Six hybridomas found to produce MAbs to heparin lyase I were designated M-1A, M2-B7, M2-A9, M-32, M-33, and M-34 (Lohse, 1992). Protein concentrations of the MAb solutions were determined using BCA Protein Assay Reagents from Pierce (Rockford, IL, U.S.A.).

Buffers for immunoassay procedures

Nitrocellulose membranes, Goat anti-Mouse IgG (H + L) Horseradish Peroxidase (HRP) Conjugate, Tris {hydroxymethyl} aminomethane (Tris), gelatin, Tween-20 and HRP Color Development Reagent (4-chloro-1-naphthol) were purchased from Bio-Rad (Richmond, CA, U.S.A.). Tris buffered saline (TBS) was 20 mM Tris containing 500 mM sodium chloride, pH 7.5. Blocking solution was 3.0% gelatin in TBS. Tween-20 wash solution diluted in TBS (TTBS) was 0.05% Tween-20 in TBS. Antibody buffer was 1% gelatin in TBS. HRP color development solution was made by mixing 60 mg HRP Color Development Reagent in 100 ml methanol at 0°C with 0.015% H_2O_2 in TBS just prior to use.

Immunoassay analysis of heparin lyases using monoclonal antibodies

Dot-blotting immunoassay techniques were conducted as recommended in the Bio-Rad Immun-Blot Assay protocol (Bio-Rad, Richmond, CA, U.S.A.). Briefly, nitrocellulose membranes were cut to 2 × 3 cm pieces and 1 × 1 cm squares were drawn on the membranes using a soft pencil. The membranes were soaked in TBS for 15 min and air dried on filter paper for 15 min. Various concentrations of the heparin lyase (1 μl in TBS) were placed in the center of each square and the membrane was air dried for 15 min. Then the membrane was immersed in blocking solution for 1 hr to coat the remaining hydrophobic sites. This was washed 4 times in TTBS (two quick rinses, then two 5 min agitations) and then soaked overnight in a solution of MAb 0.2% (v/v) in antibody buffer. Then the membranes were washed 4 times with TTBS and added to a solution of Goat anti-Mouse-HRP (0.1% in antibody buffer) for 4 hr with gentle agitation. The membranes were washed 4 times with TTBS, then twice with TBS. HRP color development solution was added to the membranes and when the purple bands were clearly visible, the development was stopped by placing the membranes in distilled water. The membranes were then dried on filter paper for 15 min and covered with aluminum foil to protect from light. Photographs of the membranes were taken.

Electrophoresis

Materials. Electrophoresis was performed using a Mini-Protean II electrophoresis cell from Bio-Rad (Richmond, CA, U.S.A.). Acrylamide and *N,N′*-methylene bisacrylamide were from International Biotechnologies Inc. (New Haven, CT, U.S.A.) or used as a prepared 40% acrylamide solution that is 37.5 acrylamide: 1 *N,N′*-methylene bisacrylamide (Fischer Scientific, Fairlawn, NJ, U.S.A.). Tris {hydroxymethyl} aminomethane (Tris) was from Bio-Rad (Richmond, CA, U.S.A.). *N,N,N′,N′*-Tetramethylethylenediamine (TEMED) was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Ammonium persulfate (APS) and glacial acetic acid were from Mallinckrodt Inc. (Paris, KY, U.S.A.). Urea and glycerol were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Sodium dodecyl sulfate (SDS) was from BDH Chemicals, Ltd. (Poole, England). Naphthol red was from Sigma Chemical Co. (St Louis, MO, U.S.A.). 2-β-mercaptoethanol was from EM Science (Gibbstown, NJ, U.S.A.). Bromophenol blue was from MCB Manufacturing Chemists, Inc. (Cincinnati, OH, U.S.A.). Molecular Weight Standards and Rapid Coomassie Stain were from Diversified Biotech (Newton Centre, MA, U.S.A.).

SDS-polyacrylamide gel electrophoresis (PAGE)

Heparin lyases I, II, III and *Flavobacterium heparinum* cell homogenate were analyzed using SDS-PAGE as previously described (Lohse, 1992; Lohse *et al.*, 1992). Separating gels (12% acrylamide, 0.1% SDS) were prepared by mixing 4.35 ml distilled water, 2.5 ml of 1.5 M Tris, pH 8.8, 0.1 ml of 10% SDS and 3.0 ml of a commercially prepared solution of 37.5 acrylamide: 1 *N,N′*-methylene bisacrylamide (Fischer Scientific, Fairlawn, NJ, U.S.A.). This solution was degassed under vacuum for at least 15 min. Next, 50 μl of APS (10%) and 5 μl of TEMED were added to the monomer solution to initiate polymerization. The gel solution was quickly poured between two glass plates separated by 0.75 mm spacers, overlaid with distilled water saturated *n*-butanol and allowed to polymerize at 25°C for 60 min.

Stacking gel was prepared by mixing 6.4 ml distilled water, 2.5 ml 0.5 M Tris, pH 6.8, 1.0 ml acrylamide/Bis solution (Fischer Scientific), 0.1 ml of 10% SDS, degassed and then 50 μl APS (10%) and 10 μl TEMED were added. The *n*-butanol was removed from the separating gel, the gel was rinsed with distilled water and the stacking gel solution was carefully added to the top of the separating gel. A well-forming electrophoresis comb was inserted in the stacking gel prior to polymerization. The stacking gel was allowed to polymerize for 60 min and the well-forming comb was removed just prior to loading of the samples.

Sample buffer was prepared by mixing 4.0 ml distilled water, 1.0 ml 0.5 M Tris, pH 6.8, 0.8 ml glycerol, 1.6 ml SDS (10%), 0.4 ml 2-β-mercaptoethanol and 0.2 ml bromophenol blue (0.05% w/v). Samples and molecular weight standard markers for electrophoresis were diluted 1:4 in sample buffer and heated for 4 min at 100°C just prior to loading into the wells formed earlier in the stacking gel. Running buffer (0.025 M Tris, 0.2 M glycine, 0.1% SDS, pH 8.3) was carefully overlaid on the stacking gel and the electrophoresis was conducted at a constant voltage of 200 V until the bromophenol blue marker moved to within 0.3 cm of the bottom of the gel (typically about 45 min). Following electrophoresis, the gels were either electro-transferred to nitrocellulose membranes (see below) or were fixed with 12.5% TCA for 15 min and stained with Rapid Coomassie Stain for 45 min followed by destaining with a 7.5% methanol/5% acetic acid solution.

Urea/acetic acid-PAGE

In some experiments urea/acetic acid-PAGE system (Panyim and Chalkley, 1969) was used instead of SDS-PAGE to compare the effects of SDS on the capacity of the MAbs to detect the heparin lyases in Western blots. Stock solutions used in the preparation of the urea/acetic acid-PAGE gels were prepared as follows. A 60% acrylamide solution was

Table 1. MAb concentrations

MAb	(mg/ml)*
M-32	4.9
M-33	4.5
M-34	4.1
M-1A	4.2
M2-A9	4.4
M2-B7	4.8

*MAb solution protein concentration determined by BCA protein assay (Pierce).

prepared by dissolving 60 g acrylamide and 0.4 g *N,N'*-methylene bisacrylamide in 100 ml of distilled water. A 43.2% acetic acid/TEMED stock solution was prepared by mixing 43.2 ml acetic acid, 4.0 ml TEMED and 52.8 ml distilled water. APS/urea was prepared by dissolving 5 mg APS in 25 ml of 10 M urea.

The urea/acetic acid-PAGE gels were formed by mixing 4.0 ml of 60% acrylamide solution, 3.0 ml 43.2% acetic acid/TEMED and 2.0 ml distilled water. This solution and the APS/urea solution were degassed for 15 min. 15 ml of the APS/urea was added to the acrylamide monomer solution, mixed and carefully poured between two glass plates separated by two 0.75 mm spacers. A well-forming electrophoresis comb was inserted into the top portion of the gel and the gel was allowed to polymerize for 60 min.

The heparin lyases were diluted 1:4 in urea/acetic acid sample buffer. This sample buffer was prepared by mixing 520 μl acetic acid, 1.0 ml glycerol, 1.0 mg naphthol red, and 6.0 g urea in distilled water that was brought to a final volume of 10 ml. The well-forming comb was removed and samples were loaded into wells and overlaid with running buffer (0.9 M acetic acid). Electrophoresis was conducted at a constant current of 20 mA for 3 hr (prefocusing of the gel) and then at 10 mA until the naphthol red moved to about 0.3 cm from the bottom of the gel (about 3 hr).

Electro-transfer of heparin lyases from acrylamide gels to nitrocellulose membranes

Semi-dry transblotting was conducted using a SemiPhor Transfer Unit (TE-70) from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). Electro-transfer of the heparin lyases from the SDS-PAGE or urea/acetic acid-PAGE to nitrocellulose membranes was accomplished using semi-dry transblotting techniques as described previously (Al-Hakim and Linhardt, 1990) except that 50 mM sodium phosphate, pH 6.8 was used as the transfer buffer. Transfer was accomplished in 40 min at 8 V.

Western blot detection of the heparin lyases using the monoclonal antibodies

Heparin lyases on the nitrocellulose membranes were detected using Western blotting techniques exactly as described above for dot-blotting immunoassay procedures.

Effects of SDS on detection of monoclonal antibodies

The effects of SDS and 2-β-mercaptoethanol on the immunodetection of the heparin lyases by MAbs M-32 and M-33 were examined. Dot-blotting immunoassays of heparin lyases I and II were performed as described earlier except that the heparin lyases were dissolved in solutions containing SDS and/or 2-β-mercaptoethanol in the same proportions used in SDS-PAGE analysis prior to blotting on the nitrocellulose membrane.

RESULTS AND DISCUSSION

Heparin lyases I, II, and III were isolated and purified from *Flavobacterium heparinum* (Lohse, 1992). MAbs to heparin lyase I were prepared by isolating B lymphocytes from heparin lyase I sensitized mice and fusing these cells with a myeloma cell line to form a MAb producing B cell hybridoma. The final concentrations of the MAbs in the culture solution are summarized in Table 1.

The reactivity of each of the six MAbs toward the three heparin lyases was examined. Varying amounts of each of the three heparin lyases were spotted on nitrocellulose membranes and detected using the anti-heparin lyase MAbs followed by addition of Goat anti-Mouse IgG-HRP and color development of the immune conjugates. Table 2 summarizes the lowest levels of each of the heparin lyases that were detected by immunoassay procedures. As seen in Table 2, the MAbs have a broad range of sensitivities toward immunodetection of the three heparin lyases. For instance, M2-A9 and M2-B7 can detect as little as 10 pg of heparin lyase II, whereas M-32, M-33 and M-34 require the presence of 1 μg of heparin lyase III in order to detect that lyase.

These data suggest that MAbs could be used to distinguish between heparin lyases I and II when the two are present together such as in a *Flavobacterium heparinum* cell homogenate. Specifically, M-32 can detect levels of heparin lyase I that are 10 times lower than heparin lyase II. Conversely, M2-A9 and M2-B7 can detect levels of heparin lyase II that are ten times lower than heparin lyase I. M-33, M-34 and M-1A cannot be used to distinguish between heparin lyases I and II. Furthermore, all six of the MAbs are able to detect much lower levels of heparin lyases I and II than of heparin lyase III, thus permitting distinction between heparin lyase III and heparin lyases I or II. Distinction between heparin lyases I and II is important because both enzymes can act on heparin and

Table 2. MAb detection of heparin lyases on nitrocellulose membranes*

MAb	Heparin lyase I	Heparin lyase II	Heparin lyase III
M-32	10 ng	100 ng	1 μg
M-33	10 ng	10 ng	1 μg
M-34	10 ng	10 ng	1 μg
M-1A	100 pg	100 pg	10 ng
M2-49	100 pg	10 pg	10 ng
M2-B7	100 pg	10 pg	10 ng

*The minimum amount of each heparin lyase detectable by each of the six MAb using dot-blotting immunodetection.

heparan sulfate and therefore they are not easily distinguished based on their substrate specificity (Linhardt *et al.*, 1990a).

Western blot analysis of the heparin lyases

The three heparin lyases and *Flavobacterium heparinum* cell homogenate samples were analyzed on SDS-PAGE followed by Western blotting immunodetection. Figure 1(B) contains a typical SDS-PAGE gel of the three heparin lyases stained with Coomassie Blue along with molecular weight markers. The ability of MAbs to detect heparin lyases was examined by running the three heparin lyases and *Flavobacterium heparinum* cell homogenate through six SDS-PAGE gels followed by Western blotting immunodetection of the gel contents. Heparin lyase I (18 ng), heparin lyase II (570 ng), heparin lyase III (1.63 μg) and cell homogenate (87 ng) were loaded on each gel. The developing time used for detection on the nitrocellulose membrane containing M-34, M1-A, M2-A9 and M2-B7 were 20, 10, 15 and 40 min respectively. Four of the MAbs (M-34, M-1A, M2-A9 and M2-B7) were able to detect purified heparin lyases I, II and III as well as heparin lyases present in the *Flavobacterium heparinum* cell homogenate. Two MAbs (M-32 and M-33) were not able to detect either the purified heparin lyases or cellular proteins in the Western blots.

Thus, we sought to determine the reagent in the SDS-PAGE system that was responsible for destroying the ability of M-32 and M-33 to immunodetect the heparin lyases. Dot-blotting immunoassays of the heparin lyases using M-32 and M-33 were used to evaluate each component in the SDS-PAGE system. Heparin lyases I and II, in the presence or absence of SDS and/or 2-β-mercaptoethanol, were blotted on nitrocellulose membranes and examined using dot-blotting immunoassay techniques. The MAbs were unable to detect the lyases when SDS was present, thus demonstrating that SDS was responsible for the reduction of sensitivity of these two MAbs during the Western blotting procedures. This experiment suggests that M-32 and M-33 must be recognizing an epitope on the lyases that requires secondary conformation such as a folded structure present in all three heparin lyases that is destroyed by SDS denaturation.

To further demonstrate that the SDS was responsible for the diminished reactivity of M-32 and M-33 toward the heparin lyases, the three heparin lyases and *Flavobacterium heparinum* cell homogenate were analyzed using the urea/acetic acid-PAGE followed by Western blotting immunodetection with M-32 and M-33 to detect the lyases in this system. The sensitivity of detection was markedly reduced. Heparin lyase I (2.7 μg), heparin lyase II (3.4 μg), heparin lyase III (4.7 μg) and cell homogenate (7.7 μg) were detectable. Thus, SDS is the agent primarily responsible for the reduced reactivity of MAbs M-32 and M-33 toward the heparin lyases. All six MAbs are able to detect all three heparin lyases, in either the purified or the native form, when analyzed using PAGE followed by Western blotting immunodetection.

When we prepared the MAbs originally, our objective was to use the MAbs to rapidly distinguish between the three lyases. We anticipated that at least one of the six MAbs would specifically detect a single heparin lyase. Such a MAb would enable the detection of that lyase in a complex mixture of heparin lyases such as a cell homogenate. The dot-blotting and Western analyses revealed that all of the MAbs are able to detect all three lyases. This observation suggests that these three heparin lyases are remarkably similar in structure since they share six common epitopes. Peptide mapping of these three enzymes (Lohse, 1992) demonstrates a number of common peptide fragments and suggests that these may be located at the highly immunogenic regions within the three heparin lyases. The sensitivities of individual MAbs toward each of the lyases in the dot-blotting analyses varied greatly, thus offering the potential to use the dot-blotting analysis to distinguish between the three lyases.

Use of PAGE (SDS or urea/acetic acid) required much more protein than dot-blotting procedures and the sensitivities of the MAbs toward each of the lyases were different than those seen in the dot-blotting analyses, probably due to alterations of secondary structure during the PAGE and transfer steps. Thus, detection of heparin lyases using MAbs is most efficiently conducted by use of dot-blotting techniques as described here. Furthermore, all six MAbs were able to detect all three lyases that were present in *Flavobacterium heparinum* cell homogenate, thus offering the potential that these MAbs could be used to rapidly demonstrate the presence of heparin lyases in cell homogenate. To be beneficial in lyase purification, these MAbs must first be immobilized and their binding avidity to the heparin lyases assessed. These studies are currently underway.

Taken together, the results described here demonstrate that MAbs can be used to detect heparin lyases I, II and III in either their purified state or when present together in a solution of homogenized Flavobacterial cells. These MAbs can also be used in dot-blotting analyses of the lyases to distinguish between the three lyases based on their different sensitivity for each of the three lyases. The MAbs and the immunodetection procedures described here represents a useful tool for the study and purification of heparin lyases.

SUMMARY

Six monoclonal antibodies were obtained from hybridomas formed by fusion of mouse myeloma cells with mouse lymphocytes that had been obtained from heparin lyase I sensitized mice. The monoclonal antibodies were examined for their ability to detect heparin lyases I, II and III in dot-blotting immunodetection. All six monoclonal antibodies detected all

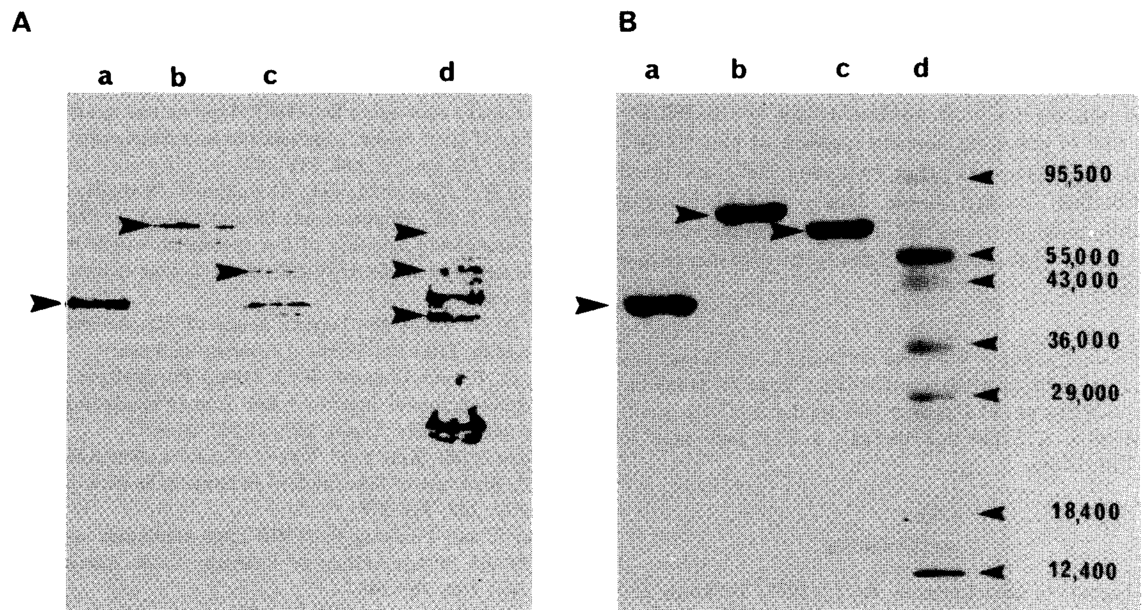

Fig. 1. (A) Western blot of SDS-PAGE gel using M2-A9. (a) Heparin lyase I, (b) heparin lyase II, (c) heparin lyase III, (d) *Flavobacterium heparinum* cell homogenate. Arrows indicate bands of interest. This analysis demonstrates the ability of this MAb to detect the presence of heparin lyases that are either purified or present in homogenized cellular material. (B) SDS-PAGE analysis of purified heparin lyases. (a) Heparin lyase I, (b) heparin lyase II, (c) heparin lyase III, (d) molecular weight markers. Arrows indicate bands of interest.

three heparin lyases, but the individual antibodies varied in their reactivity toward each of the three lyases, thus offering the potential for use of these antibodies to distinguish between the three lyases. The monoclonal antibodies were also used to detect the three heparin lyases in either the purified state or when present in *Flavobacterium heparinum* cell homogenate during SDS-PAGE analysis followed by Western blotting. We observed that SDS interfered with the reactivity of two of the antibodies toward the three lyases, indicating that these two antibodies must be recognizing a secondary structure in the lyase. When urea/acetic acid-PAGE analysis was used instead of SDS-PAGE and followed by Western blotting, the antibodies were able to detect all three lyases, further demonstrating that the SDS was responsible for the reduction in reactivity of the antibodies toward the lyases in the SDS-PAGE system. We conclude that the monoclonal antibodies we produced are capable of detecting heparin lyases I, II and III and that differences in the reactivity of individual antibodies toward each lyase in dot-blotting immunodetection can aid in distinguishing between the lyases when purifying them from *Flavobacterium heparinum*.

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