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Immunochemical characterisation of species-specific antigens in bovine crude heparin

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Abstract

In order to develop immunoassays for the control of the species origin of crude heparins, polyclonal antisera were produced in rabbits against samples obtained from the last purification steps of bovine intestinal crude heparin. The reactivity of the antisera was analysed by agar gel double immunodiffusion, immunoelectrophoresis, crossed and line immunoelectrophoresis. Up to 13 antigenic components were detected in the effluents of the ion-exchange chromatographic step, and three in the final crude heparin. The major and most anodic antigen (Ag1) was recovered in bovine crude heparin purified by the two different industrial processes. This bovine specific antigen was found in high concentrations in lung, liver, small intestine, spleen and kidney. It displayed an apparent molecular weight of 45 kDa by size-exclusion chromatography. Though the identification of Ag1 is not yet fully elucidated, a single radial immunodiffusion assay has been developed for the quantification of this antigen, allowing the detection of 6 p 1000 bovine crude heparin in porcine heparin (50 mg/ml) after 2 h diffusion.

Keywords: Heparin; Bovine spongiform encephalopathy; Bovine antigen; Immunochemical detection

1. Introduction

Heparin is a polydisperse mixture of linear, highly sulphated polysaccharides composed of repeating $\alpha 1 \rightarrow 4$ linked uronic acid residues (either D-glucuronic acid or L-iduronic acid) and D-glucosamine. As it contains a specific pentasaccharide sequence [1] that binds and potentiates the activity of antithrombin III (a serine protease inhibitor) it has been used for over half a century as an anticoagulant and antithrombotic drug. Approximately 500 million heparin doses are used worldwide each year for this purpose and the large number of favourable biologic activities unrelated to anticoagulation leads to a constant growing interest for new therapeutic applications [2,3].

In spite of the emergence of recombinant biopolymeric drugs, commercial heparin is still purified from animal sources. It is exclusively found in mast cells and can be extracted from various highly vascularised tissues [4], usually intestinal mucosa or lung from pigs, cattle and in a

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lower extent from sheep. The outbreak of bovine spongiform encephalopathy (BSE) and the accumulation of evidences suggesting that the BSE and the new variant of Creutzfeldt-Jakob disease prions are identical [5–7] have led Health Authorities to ban ruminant heparins for human therapy. Accordingly, it became urgent to develop analytical methods intended to confirm the species origin of heparin. The availability of such methods should represent a powerful tool to control the application of this restriction and to certify the biological safety of the derived therapeutic drugs.

The control of the animal origin of pure heparin batches has been studied by ^{13}C NMR [8–10] and HPLC [10–13]. Differences in acetylation and sulphation patterns of unsaturated disaccharides have been described between bovine, ovine and porcine heparinase digests. However, intra-species variations in heparin structure and enzymatic or chemical desulphation have been observed during the storage of the mucosa and the purification process. Consequently, misinterpretation in the determination of pure heparin origin can occur [11,13] and the detection level is usually $\geqslant 5\%$. The limitations of these physico-chemical methods underline the need of complementary methods to control the raw materials used for pure heparin production.

To ascertain the porcine origin of intestinal mucosa, two sensitive immunoassays have been recently reported [14]. The enzyme-linked immunosorbent assay (ELISA) has enabled the detection of 10 ppm of bovine intestinal mucosa in porcine intestinal mucosa. The single radial immunodiffusion assay (SRID), more convenient for routine control, has been found suitable to detect 3 p 1000 bovine, ovine or caprine intestinal mucosa in porcine intestinal mucosa.

Up to now, no method has been reported for the control of crude heparins. Since these products are not fully purified, species-specific impurities could remain. The presence of these impurities could open the possibility to develop specific immunoassays to control the animal origin of these raw materials.

Our objective was therefore to assess, using immunochemical methods, the antigenicity and species-specificity of the contaminants of bovine crude heparin. One of them, called Antigen 1 (Ag1), was shown to be bovine specific and mostly present in intestinal mucosa, lung and liver. Specific quantification of this antigen by SRID enabled to detect 6 p 1000 bovine crude heparin in porcine heparin (50 mg/ml).

2. Materials and methods

2.1. Chemicals

Freund's complete and incomplete adjuvants, bovine serum albumin (BSA) and vitamin B12 were purchased from Sigma Chemical Company (St Louis, MO). Agar Noble (Difco) was supplied by Serlabo (Bonneuil-sur-Marne, France), and agarose for electrophoresis by Labosi (Oulchy-le-château, France). BCA protein assay reagent was purchased from Pierce (Rockford, IL) and glutaraldehyde from Serva (Heidelberg, Germany). Crude porcine mucosa heparin was supplied by Valori-5 (Ploërmel, France).

2.2. Crude heparins preparation

Bovine (BovHep1), ovine and caprine crude heparins were purified from proteolytically degraded hashed gut or lung by adsorption on macroporous anion-exchange resins (process 1) essentially as described by Griffin et al. [15]. Briefly, the tissues were sampled at a local slaughterhouse, homogenised and then proteolysed at 55–65 °C with a proteolytic enzyme from *Bacillus subtilis* (Alcalase 2.4 L, NovoNordisk, Bagsvaerd, Denmark) until total liquefaction. After filtration, sodium chloride was added up to 1 M and incubated 15 h with a macroporous strong anion-exchange resin (Lewatit MP500A, Bayer, Pittsburgh, PA). The resin was thoroughly washed with 1 M NaCl and the adsorbed material was eluted with 3 M NaCl. Heparin was then selectively precipitated from the eluted materials (EM) with 50% methanol and vacuum dried. Portions of EM and of supernatant of the alcohol precipitation step (SAP) were concentrated by ultrafiltration (Sartorius, molecular mass cut-off 10 kDa) under nitrogen pressure and then extensively dialysed against Tris–HCl buffer (pH 7.5; 0.1 M).

A sample of bovine crude heparin (BovHep2) was also obtained by proteolysis of hashed gut as in process 1, heat coagulation at 90 °C and cetylpyridinium chloride complexation according to Scott [16] (process 2).

2.3. Protein assay

Protein content of crude heparins, EM and SAP was determined with the BCA Protein Assay Reagent (Pierce) according to the microtiter plate protocol described by the manufacturer. BSA was used as a standard.

2.4. HPLC analysis

Samples (0.1 ml) of EM, SAP and BovHep1 (20 mg/ml) were submitted to size-exclusion chromatography using a Zorbax GF250 column (Dupont de Nemours, Wilmington, DE) equilibrated and eluted with phosphate buffer (pH 7.8; 0.1 M; 2 M NaCl). Elution was performed at 1 ml/min using an HPLC equipment (Pump 420, Detector 430; Kontron Instruments, F-78180 Montigny-les-Bretonneux, France). A bovine whey supplemented with BSA (3 mg/ml, final concentration) and vitamin B12 (1 mg/ml, final concentration) was used for the column calibration.

2.5. Antisera

Rabbit antisera were produced against 50 mg/ml BovHep1 in phosphate buffered saline (pH 7.4; 0.01 M) (PBS) and 10-fold concentrated EM and SAP. For each sample, four rabbits were immunised at monthly intervals by multiple intradermal injections of antigen–adjuvant mixture prepared by emulsifying 1 ml of antigen samples with 1 ml of complete (first injection) or incomplete (booster injection) Freund's adjuvant [17]. Animals were bled 7 and 9 days after each booster injection.

2.6. Absorption of anti-BSA antibodies

Antibodies reacting with BSA in the anti-BovHep1 antiserum were absorbed using BSA beads obtained by glutaraldehyde polymerisation essentially as described by Avrameas and Ternynck [18]. Briefly, BSA (150 mg) was dissolved in 5 ml PBS. The pH was adjusted to 5.0 with acetate buffer (pH 5.0; 2 M). Glutaraldehyde (1.5 ml of a 2.5% v/v solution in distilled water) was added dropwise under magnetic stirring and the solution allowed to polymerise without stirring until complete gel formation. The polymer was then broken up with a sorvall Omnimixer in phosphate buffer (pH 7.4; 0.1 M) to obtain a suspension of small beads which were vacuum degassed and centrifuged at $2000 \times g$ for 5 min. A second treatment was carried out and the pellet resuspended for 15 min in 50 ml of 0.1 M lysine in 0.15 M NaCl. The suspension was then washed twice in 50 ml of PBS with centrifugation at $2000 \times g$ for 5 min. The antiserum (5 ml) was incubated with rotating stirring for 1 h at room temperature together with 5 ml of sedimented beads. After centrifugation at $2000 \times g$ for 15 min at 4 °C the supernatant was tested for BSA reactivity by double immunodiffusion in 1.2% agar Noble.

2.7. Tissues extracts

Bovine small intestine, lung, spleen, kidney, longissimus dorsi and porcine lung were freshly collected in a local slaughterhouse, cut into approximately 10 g pieces and chopped in a robot coupe commercial processor (Moulinex, France). A typical extraction consisted of adding 10 ml of PBS to 5 g of the chopped tissue followed by high speed blending for 15 s with an Ultraturrax IL-X-1020. The suspension was centrifuged at $15\,000 \times g$ for 15 min at 4 °C. The aqueous phase was then adequately diluted in PBS for Ag1 quantification by SRID.

2.8. Immunoprecipitation techniques

Ag1 concentrations in tissues extracts and heparins were determined by SRID assay [19] using 1.8 mm thick agar plates (7×11 cm^2) containing 1.2% agar Noble in veronal buffer (pH 7.3; 0.05 M) and suitable quantities of anti-BovHep1 rabbit antiserum absorbed against BSA. Circular wells (1.5 mm diameter) were punched out in the gel and filled with 3 μl portions of adequately

diluted samples or 3 μl of known concentrations of BovHep1 in PBS. After 24 h diffusion at 37 °C in a humidified chamber the plates were immersed in 2% (v/v) acetic acid for 1 min and rinsed for 15–30 min with 0.15 M NaCl. The diameters of the ring-shaped precipitates were measured using dark-field oblique illumination and a magnifying video camera system [20]. Standard curves were constructed by plotting the diameter of the ring versus the square root of BovHep1 concentration.

Immunoelectrophoresis was carried out on 7 × 11 cm^2 plates using 1.2% agar Noble gel in barbital–HCl buffer (pH 8.6; 0.025 M). Tanks were filled with barbital–HCl buffer (pH 8.6; 0.05 M). Migration was allowed to run at 6 V/cm for 50 min.

Fused rocket immunoelectrophoresis was performed according to Svendsen [21] using suitable quantities of antisera in 1.2% agarose. Electrophoresis was run at 2.5 V/cm for 180 min.

Line immunoelectrophoresis was performed according to Kroll [22] using 1.2% agarose. Antigens and/or antisera to be compared were moulded into adjoining gel sections. Electrophoresis was run at 2.5 V/cm for 180 min.

Crossed immunoelectrophoresis was done according to Weeke [23]. Electrophoresis was run at 3.5 V/cm for 60 min for the first dimension and at 2.5 V/cm for 120 min for the second dimension.

After immunodiffusion or immunoelectrophoresis the gels were washed in 0.15 M NaCl containing 0.1% sodium azide for 24 h at 4 °C under continuous stirring to remove non-precipitated proteins. Then they were slipped on a GelBond® film (Pharmacia Biotech), dried and stained in a 0.12% Coomassie Brilliant Blue R250 water/ethanol/acetic acid solution (65:30:5, v/v/v). The precipitates obtained by line electrophoresis were scanned on a photodensitometer (Vernon, Paris, France).

3. Results

3.1. Physico-chemical characterisation of the BovHep1, EM and SAP used for immunisations

Protein contents of EM and SAP dialysed against Tris–HCl buffer (pH 7.5; 0.1 M) and of BovHep1 (2 mg/ml in the same buffer) were respectively 37.7, 9.7 and 23.8 μg/ml. By UV spectrophotometry, no maximum of optical density was observed between 210 and 310 nm. Optical density for EM, SAP and BovHep1 were 0.240, 0.063 and 0.820 respectively at 280 nm and 1.200, 0.275 and 5.670 respectively at 214 nm. As a consequence of this weak protein content, EM and SAP were 10-fold concentrated by ultrafiltration prior to the immunisation of the rabbits.

Size-exclusion chromatographic elution profiles of EM, SAP and BovHep1 are depicted in Fig. 1. Only few contaminants with molecular mass higher than 10 kDa were detected in EM (Fig. 1b). The elution profile of the concentrated EM showed a high decrease in the low molecular weight contaminants and an increase in protein contaminants detected at 280 nm in the 10–15 kDa and 40–50 kDa fractions (Fig. 1c). The elution profile of the concentrated SAP showed a weak optical density at 214 and 280 nm (Fig. 1d). The elution profile of BovHep1 displayed a first peak of excluded material, a second major peak in the 100–150 kDa and a third peak in the 40–50 kDa zones (Fig. 1e). Only few contaminants with molecular mass lower than 13 kDa were observed, both at 280 and 214 nm.

3.2. Immunochemical characterisation of antisera against EM

The specificity of the rabbit antisera against bovine EM (anti-EM) was firstly analysed by agar gel double immunodiffusion. Three to four precipitates were observed against bovine EM (Fig. 2a). One of them was also identified in BovHep1 (Fig. 2b). Since this precipitate was the nearest to the antiserum hole, this antigen was referred to as Ag1. This antigen was not recognised by anti-EM no. 3 (Fig. 2a). It appeared to be non-specific of the process and of the tissue used for heparin extraction since it was also identified in BovHep2 (Fig. 2c) and in bovine lung EM (not shown).

When tested against caprine EM and caprine crude heparin (Fig. 2d) the anti-EM revealed one strong arc antigenically unrelated to Ag1. Very

slight precipitates were observed against ovine EM and no precipitate was obtained with ovine crude heparin (results not shown). The antisera never reacted against porcine crude heparin, porcine lung EM or porcine intestinal EM (see further in Fig. 7b).

Using immunoelectrophoretic analysis, the anti-EM revealed one major arc in a more anodic position than BSA and 1–2 minor arcs. Consequently, the antigen holes were then punched out in the cathodic side of the agarose plate. The major arc was not revealed by rabbit no. 3 and was thus identified as Ag1 (Fig. 3a).

The highly negative charge of the antigenic contaminants detected by the rabbit antisera allowed us to use more discriminant techniques based on antigen electrophoresis through antiserum-containing gel. By fused rocket immunoelectrophoresis, eight antigens were detected in bovine EM. The most anodic of these precipitates displayed strong antigenic community with the sole precipitate detected in BovHep1 and was thus identified as Ag1 (Fig. 3b). Using the powerful resolution of crossed immunoelectrophoresis, 11 individual precipitates were detected in bovine EM (Fig. 4a). In BovHep1 we observed a major

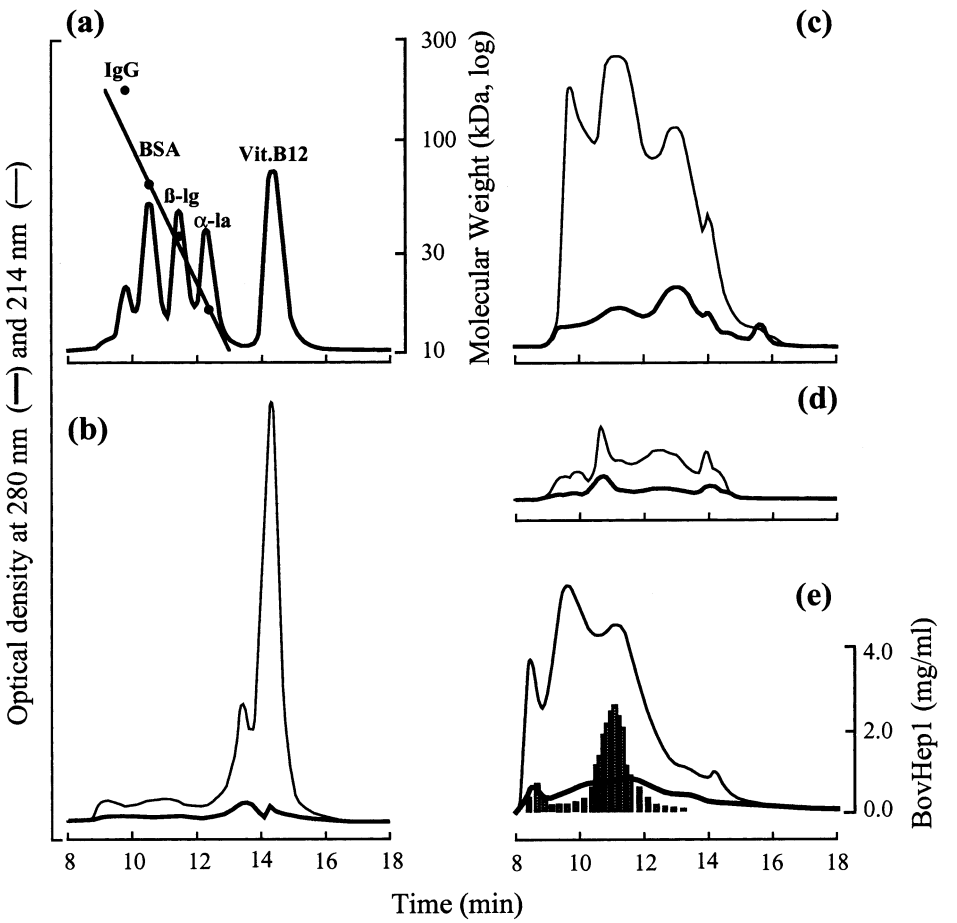

Fig. 1. Elution profiles from size-exclusion-HPLC on a Zorbax GF250 column. (a) Column calibration with a bovine whey supplemented with BSA and vitamin B12. The molecular weight (log scale) of IgG, BSA, β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were plotted versus their retention time; (b) non-concentrated bovine EM; (c) 10-fold concentrated bovine EM; (d) 10-fold concentrated bovine SAP; (e) BovHep1 (20 mg/ml). Ag1 immunoreactivity in the fractions was monitored by SRID and was expressed as the BovHep1 concentration that gives the same reactivity.

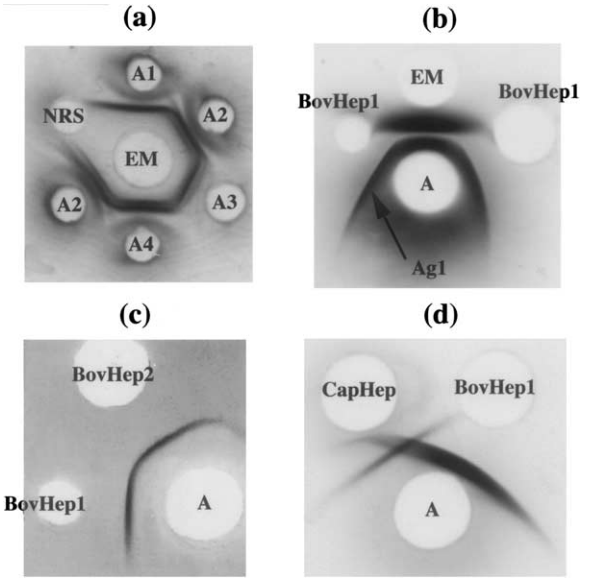

Fig. 2. Immunochemical characterisation of the rabbits anti-EM antisera by agar gel double immunodiffusion. (a) Rabbits A1–A4 against 10-fold concentrated bovine EM; NRS, normal rabbit serum; (b) Rabbit no. 1 (A) against concentrated bovine EM and 15 μl (left hole) or 60 μl (right hole) of BovHep1 (10 mg/ml in PBS). Ag1 precipitate is indicated by the arrow; (c) Rabbit no. 1 (A) against 15 μl of BovHep1 (10 mg/ml in PBS) or 60 μl of BovHep2 (10 mg/ml in PBS); (d) Rabbit no. 1 (A) against 60 μl of caprine crude heparin (80 mg/ml in PBS) or 60 μl of BovHep1 (10 mg/ml in PBS).

anodic precipitate corresponding to Ag1 and two faint precipitates with a weak migration towards the antibody-containing gel (Fig. 4b).

Antigenic relationships between the antigens recognised in BovHep1 and bovine EM by anti-EM antisera were studied using line immunoelectrophoresis. A total of 13 parallel precipitin lines were revealed with bovine EM (Fig. 5a and Fig. 5b) and essentially one precipitin line with BovHep1 (Fig. 5b). As expected, this precipitin line showed a complete antigenic identity with the most anodic line of EM and was thus identified as Ag1. Line immunoelectrophoresis was also used to compare the variability of antibody responses of the different rabbits immunised with the same antigen such as rabbits anti-EM no. 1 and no. 3 (Fig. 5b). Several precipitin lines changed their relative position owing to differences in antigen/antibody ratios. With the anti-EM no. 3, the Ag1 precipitate completely disappeared as a result of the lack of corresponding antibodies.

Densitometry of the line immunoelectrophoresis obtained with bovine EM allowed recording and quantification of the different precipitin lines. The patterns obtained are comparable to chromatographic profiles (Fig. 6a and Fig. 6b). However, in line immunoelectrophoresis the antigen concentration is related to the peak mobility instead of the classical peak height in chromatographic analysis.

3.3. Immunochemical characterisation of antisera against SAP

Using agar gel double immunodiffusion 1–3 precipitin lines were observed against SAP (Fig. 7a). One of them was also observed when testing the antisera against BovHep1 and EM and was identified as Ag1 by its complete antigenic identity with the precipitin line obtained between anti-EM no. 1 and BovHep1 (Fig. 7b).

As the antibody titers of these antisera were lower than those against EM, no further investigations were performed.

3.4. Immunochemical characterisation of antisera against BovHep1

By agar gel double immunodiffusion analysis of BovHep1, only one strong precipitate with complete antigenic identity with Ag1 was observed (result not shown). Antibody responses of the four rabbits were compared using line immunoelectrophoresis analysis. With BovHep1, only one precipitin line was observed (result not shown). However, using bovine EM, two precipitin lines were obtained with anti-BovHep1 no. 1 to 3 instead of five precipitin lines with anti-BovHep1 no. 4 (Fig. 8a). The complete fusion of the most anodic precipitin line obtained with anti-EM and anti-BovHep1 antisera allowed us to identify this line as Ag1 immunoprecipitate.

Preliminary experiments of Ag1 immunodetection by Western-blotting using the anti-BovHep1 no. 1 showed antibody reactivity against BSA. These anti-BSA antibodies were removed by immunoadsorption on beads of glutaraldehyde poly-

merised BSA. The efficiency of the absorption was then controlled by agar gel double immunodiffusion (result not shown). Using this absorbed antiserum, the second precipitin line observed in bovine EM with anti-BovHep1 no. 1 in line immunoelectrophoresis (Fig. 8a) was not revealed and thus identified as BSA (Fig. 8b).

The BSA concentration in EM and SAP was estimated using a specific SRID test with a 2 μg/ml sensitivity threshold [14]. Weak blurred ring-shaped precipitates, indicative of the partial proteolysis of the antigen, were observed for a 30-fold concentrated EM. The BSA concentration was estimated around 20 μg/ml. No precipitable BSA was found in BovHep1 analysed up to 100 mg/ml.

The BSA-absorbed anti-BovHep1 antiserum was used to develop a SRID assay for the quantification of Ag1. Under optimal conditions, a detection limit of 6 p 1000 of bovine crude heparin in porcine crude heparin (50 mg/ml) was reached after a 2-h diffusion. The parameters of the linear analytical response curve were $y = 22.4x + 35.2$ ($r = 0.999$) with y as the ring diameter of the precipitate and x the square root of the BovHep1 concentration. A RSD of 4.6% ($n = 10$) was obtained for the quantification of Ag1 in a porcine crude heparin supplemented with 2.5% bovine crude heparin.

Using this SRID assay, relative concentrations of Ag1 were determined in a panel of bovine tissues (Table 1). Lung was roughly twice as more concentrated in Ag1 as small intestine and liver. On the opposite, spleen and kidney were less than 50% of the Ag1 concentration of small intestine. No Ag1 was detected in serum and skeletal muscle.

The SRID assay was finally used to monitor the immunoreactivity of Ag1 in the fractions obtained by size-exclusion chromatography of BovHep1. An apparent molecular weight of 45 kDa was obtained by interpolation on the linear regression curve representing log(Mr) of standard proteins versus their elution volume on a Zorbax GF 250 column (Fig. 1e).

4. Discussion

Crude heparins are mixtures of complex polysaccharides with a wide distribution in molecular weight and ionic charge. Consequently, the

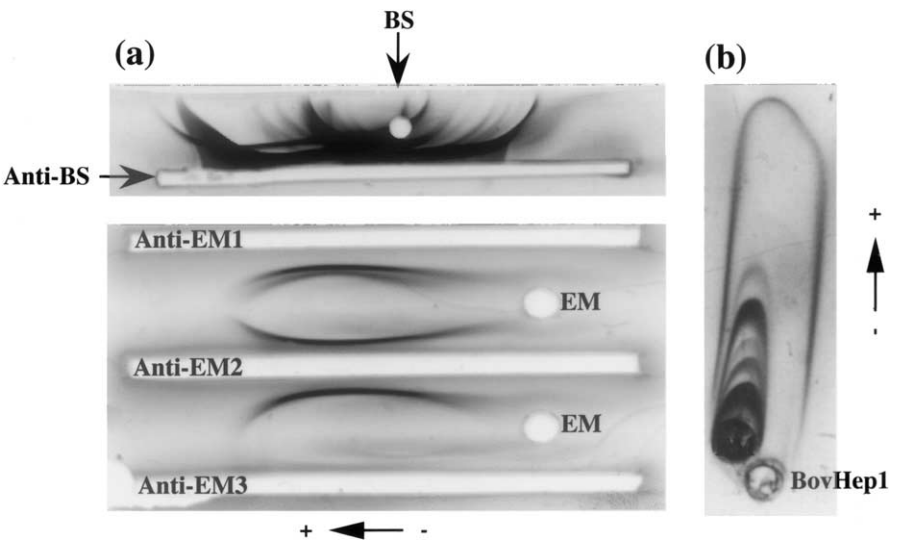

Fig. 3. Immunoelectrophoretic and fused rocket immunoelectrophoretic analyses of rabbits anti-EM antisera. (a) Immunoelectrophoretic analysis of rabbits no. 1–3 (troughs) against concentrated bovine EM (holes). Bovine serum (BS) was used as a control and was revealed using a rabbit anti-bovine serum (anti-BS); (b) Fused rocket immunoelectrophoresis of concentrated bovine EM and BovHep1 (10 mg/ml in PBS) using rabbit no. 1.

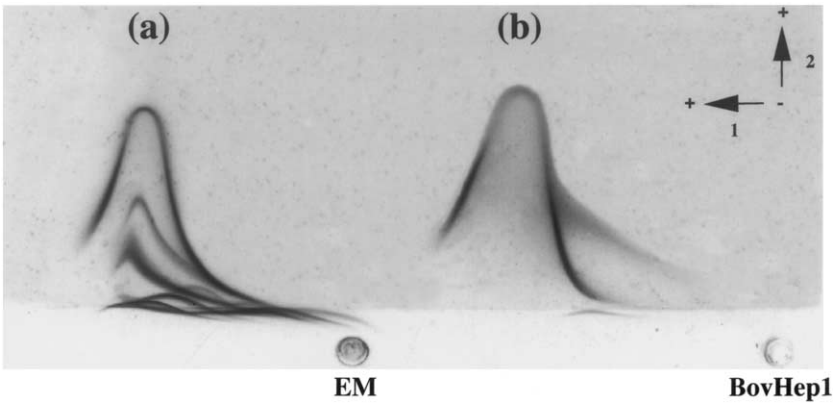

Fig. 4. Crossed immunoelectrophoretic analyses of bovine EM and BovHep1 with rabbit anti-EM antiserum no. 1. (a) 10-fold concentrated EM; (b) BovHep1 (20 mg/ml in PBS).

characterisation of traces of contaminants in crude heparin by conventional physico-chemical techniques such as size-exclusion or ion-exchange chromatography is relatively difficult. Using electrophoretic techniques such as SDS-PAGE, large smears are commonly observed and the electrophoregrams are difficult to interpret. Moreover, such physico-chemical techniques cannot detect species-specific conformational structures on a molecule. On the opposite, immunochemical tools are particularly suitable for such purposes and the well-known low immunogenicity of the heparin molecule was an advantage to produce antibodies essentially directed against the immunogenic contaminants. Since antisera raised against bovine proteins usually react poorly with the homologous porcine proteins [14,24] we could also expect to obtain antisera against bovine specific molecular structures.

In a preliminary step, we produced rabbit antisera against concentrated bovine EM and SAP since these intermediate products were expected to contain contaminants in higher concentrations than corresponding final crude heparin. The antibody responses were firstly analysed using conventional immunoprecipitation techniques such as double immunodiffusion and immunoelectrophoresis. Then, we took advantage of the negative charge of the immunogenic contaminants detected to implement more powerful techniques such as crossed immunoelectrophoresis [23] and line immunoelectrophoresis [22]. Such techniques have already proved to be very useful in the characterisation of mixtures of antigens in complex extracts [25–29] or for testing the specificity and potency of uncharacterised antisera [25]. Densitometric analysis of the precipitin lines, as proposed in this work, enabled a representation that could be compared to chromatogram obtained with high resolution chromatographic techniques (Fig. 5). Thus, each rabbit antiserum could be considered as a virtual chromatographic column with its own resolution power. By producing fusion of related precipitates (Fig. 4a), the peak positions of defined antigens were easily identified. Moreover, the modifications of antibody concentrations in the agar gel had the same apparent effect on the diagram than modification of the slope of a linear salt gradient in ion-exchange chromatography. Such an approach could be particularly useful for monitoring the purification process and could ensure better traceability of crude porcine heparin batches.

Using these techniques, a total of 13 immunogenic contaminants were revealed in bovine EM (Fig. 5), one of them being the major contaminant of crude heparin. This contaminant, called Ag1, was not tissue specific since it was detected in intestinal crude heparin as well as in lung-derived EM. Moreover, Ag1 was not process-specific since it was also found in bovine crude heparin produced by the second usual process of heparin

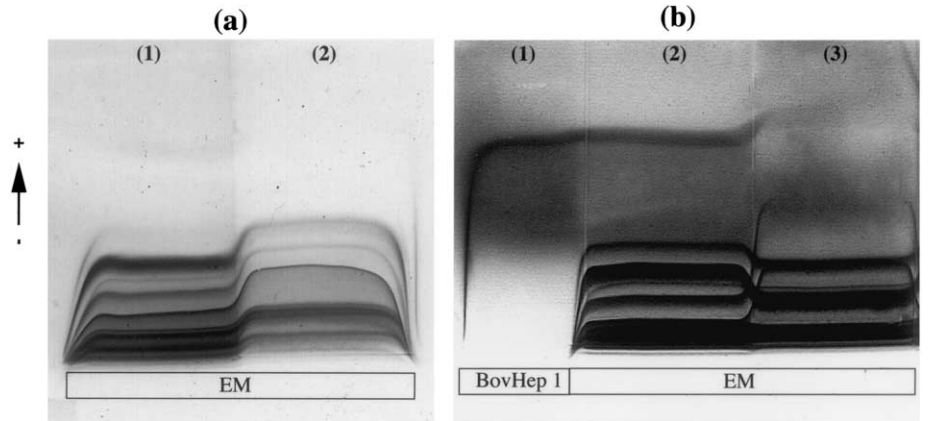

Fig. 5. Line immunoelectrophoretic analyses of bovine EM and BovHep1 with rabbits anti-EM antisera no. 1 and no. 3. (a) 10-fold concentrated EM tested against (1) rabbit no. 1 at 150 μl or (2) 75 μl/2.5 ml agarose; (b) BovHep1 (10 mg/ml in PBS) and 10-fold concentrated EM tested (1), (2) against rabbits no. 1 at 260μl/2.5 ml agarose or (3) with rabbit no. 3 at 450 μl/2.2 ml agarose.

purification (BovHep2). As expected, this antigen was not detected by means of our antisera in porcine EM or porcine crude heparin.

These results prompted us to immunise rabbits with bovine crude heparin. Most of the antisera obtained revealed a major contaminant identified as Ag1 and a second precipitin line. A clear precipitin line was also obtained when testing the antisera against BSA by double immunodiffusion (not shown), and partially proteolysed BSA was revealed in EM but not in highly concentrated (100 mg/ml) crude heparin. Thus, trace quantities of BSA fragments ($\leqslant 1$ μg/50 mg BovHep1) were sufficient to induce an antibody response in rabbits. This underlines the very high reactivity of the rabbit immune system to trace quantities ($\leqslant 20$ ppm) of immunogenic contaminants.

The presence of BSA fragments in crude heparin is not really surprising since this molecule is ubiquitous in all bovine tissues and its concentration was found to be about 2 mg/g in the small intestine [14]. This protein is quickly proteolysed during the proteolysis step of the process (Levieux and Levieux, unpublished results). However, as BSA brings a high net negative charge, the most

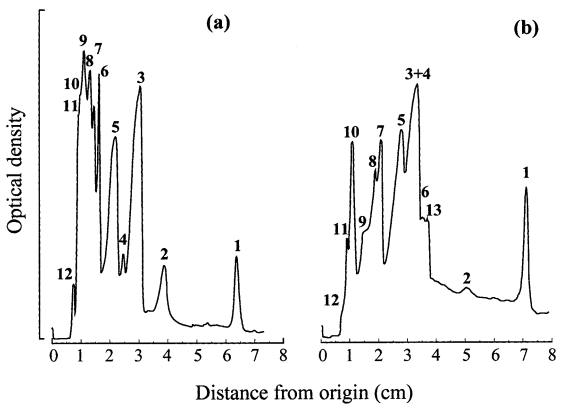

Fig. 6. Densitometric profiles of contiguous line immunoelectrophoresis performed with concentrated EM versus (a) rabbit anti-EM no. 1, or (b) rabbit anti-EM no. 2, both used at the same concentration. Peak no. 1 is the Ag1 precipitin line. Correspondence between the peaks of (a) and (b) was established using the fusion of contiguous lines as illustrated in Fig. 5b.

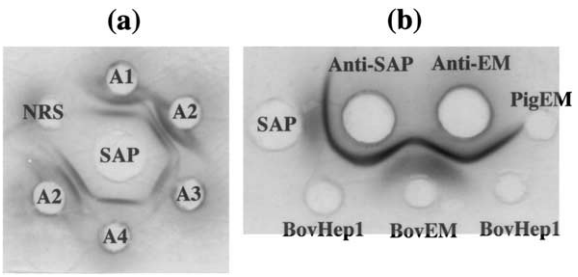

Fig. 7. Immunochemical characterisation of the rabbits anti-SAP antisera by agar gel double immunodiffusion. (a) Rabbits A1–A4 against 10-fold concentrated bovine SAP. NRS, normal rabbit serum; (b) Comparison of the reactivity of anti-SAP no. 1 and anti-EM no. 1 against SAP, BovHep1, bovine EM and pig EM.

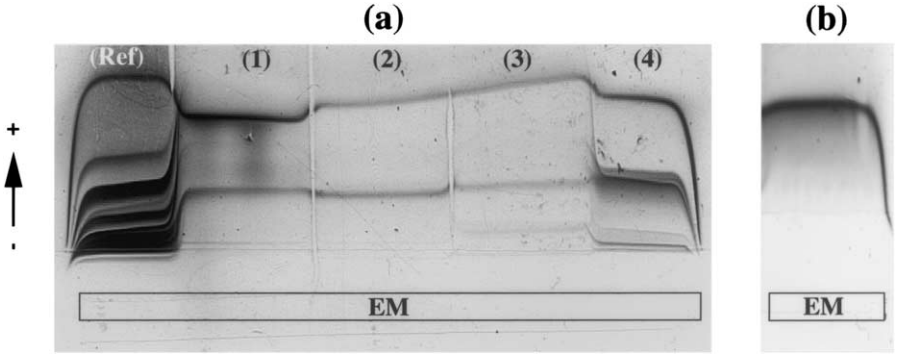

Fig. 8. Line immunoelectrophoretic analysis of the rabbit antisera against BovHep1 (a) before and (b) after absorption with BSA. (Ref): rabbit anti-EM no. 1 used as reference. (1), (2), (3), (4): rabbits anti-BovHep1 no. 1–4 respectively.

Table 1
SRID quantification of the Ag1 content of crude tissues extracts using anti-BovHep1 absorbed against BSA

Tissues	Ag1 relative quantities (%)
Small intestine	90
Lung	200
Liver	135
Spleen	43
Kidney	35
Muscle	0
Serum	0

Results are expressed as the percentage of Ag1 quantity found in a 10 mg/ml solution of bovine crude heparin (BovHep1).

anionic fragments produced by the enzymatic cleavage may behave as the heparin molecule during the ion-exchange step of the process.

Anti-BovHep1 antiserum was made monospecific by absorption of the anti-BSA antibodies on BSA beads obtained by glutaraldehyde polymerisation. This absorbed antiserum prompted us to develop a SRID technique for the specific quantification of Ag1 in crude heparin. The detection limit obtained after a 2-h diffusion was around 6 p 1000 bovine crude heparin in 50 mg/ml porcine heparin. This technique is particularly suitable for routine controls. Studies are in progress to develop a very sensitive ELISA (detection limit $\leqslant$ 10 ppm) which can be used in quality control laboratories.

Relative concentrations of Ag1 in bovine tissues were determined by means of the SRID technique. The highest values were recorded in lung, liver and intestine, tissues which have been traditionally used for heparin production since they are particularly rich in mast cells [4].

Ag1 is not yet fully characterised. As determined by size-exclusion chromatography its apparent molecular weight is 45 kDa. In view of its mobility in agar gel, its apparent pI is $\leqslant 4.0$. However, this antigen can be a very anionic substance as well as a very cationic one since heparin is well known to bind with high affinity numerous basic proteins. Thus we cannot exclude that Ag1 could be a lower molecular weight cationic substance bound to heparin and co-purified during the process. Work is in progress to purify the Ag1 and to elucidate its structure. Moreover, the monospecific anti-Ag1 antiserum obtained will allow us to carry on studies on the histological localisation of Ag1 in the bovine small intestine.

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