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Characterisation of Ag1, the major species-specific contaminant of bovine crude heparin, and its identification as an aprotinin/heparin complex

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Abstract

Heparin is a potent anticoagulant polysaccharide purified for decades from ruminants or porcine tissues. However, with the emergence of bovine spongiform encephalopathy (BSE), the source of pharmaceutical heparin is currently restricted to porcine intestinal mucosa. A major species-specific contaminant, called Ag1, has recently been identified in bovine crude heparin [Rivera et al., J. Pharm. Biomed. Anal., submitted] and used to develop an enzyme-linked immunosorbent assay (ELISA) for the species origin control of crude heparins [Levieux et al., J. Immunoassay, submitted]. In this report, we describe the different investigations, which were carried out to identify Ag1. This antigen was first localised by immunohistological studies essentially in the connective tissue of the bovine small intestine. After extraction from an intestinal extract by immuno-affinity chromatography, Ag1 was isolated as a single band by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Ag1 was then partly sequenced and identified as an aprotinin/heparin complex. Aprotinin, also known as the bovine pancreatic trypsin inhibitor (BPTI), is present with heparin in mast cells, and is very resistant to heat, pH, chemical treatments and proteolytic digestion. The stability of Ag1 towards the different treatments performed during heparin extraction process allows this protein to remain in sufficient amounts in crude heparin and makes it an ideal target for the immunochemical control of the absence of bovine material in crude heparins.

Keywords: Bovine spongiform encephalopathy; Heparin; Bovine; Aprotinin; BPTI

1. Introduction

Heparin is defined as a family of heterogeneous linear polysaccharide chains made-up of repeating units of highly sulphated disaccharides containing an uronic acid residue (either D-glucuronic acid or L-iduronic acid) and D-glucosamine [1]. Its specific binding to antithrombin III enhances the inactivation of several serine proteases of the coagulation system. This property has made heparin one of the most popular anticoagulant drugs since 1939 [2].

Heparin is synthesised in connective tissues mast cells as a serglycin proteoglycan composed of a core protein from which long polysaccharide

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chains extend [3]. As the biosynthesis continues, heparin chains are cleaved by an endo β-D-glucuronidase and stored as glycosaminoglycan heparin in the secretory granules in complexation with basic proteases [4,5].

Glycosaminoglycan heparin can be extracted from various highly vascularised mammalian tissues [6]. Its pharmaceutical form has been purified for decades from gut or lung tissues obtained from pigs, cattle and, to a lesser extent, from sheep. However, the emergence in 1986 of bovine spongiform encephalopathy (BSE) in the United Kingdom and its extension to other European countries and animal species in the 90th, have focused attention on the risk for man to be infected by pharmaceuticals derived from ruminant tissues. Consequently, to protect the public health, regulatory authorities have restricted the source of heparin for human therapy to porcine intestinal mucosa only. Accordingly, it became necessary to develop analytical methods intended to control the application of this restriction.

For the control of the animal origin of pure heparin, physico-chemical methods such as ^{13}C NMR and HPLC analysis of heparinase digests have been proposed [7–12]. These methods alone are, however, not sufficient to guarantee the absence of trace contamination by forbidden material. Consequently, a complementary and indirect approach, consisting in the development of analytical methods applied to the starting material and the crude intermediate products used for pure heparin production, has been adopted.

To ascertain the porcine origin of the intestinal mucosa collected by heparin manufacturers, two immunoassays are now available [13]: an ELISA able to detect 10 ppm of bovine tissues in porcine intestinal mucosa and a SRID, more convenient for routine control in plants, with a 3 p 1000 detection limit.

For the control of crude heparins, an immunochemical approach has been recently undertaken to detect species-specific contaminants that could remain in these intermediate products [Rivera et al., J. Pharm. Biomed. Anal., submitted]. Using rabbit polyclonal antisera in immunoprecipitation techniques, upto 13 antigenic components have been revealed in the chromatographic effluent of a bovine intestinal heparin preparation. In the final crude heparin, three of these contaminants have been recovered. The major one, called Ag1, has been found to be bovine-specific and present in pulmonary crude materials as well as in bovine intestinal crude heparins prepared using two different industrial processes. A monospecific antiserum has been produced against Ag1 and used to develop a SRID and an indirect competitive ELISA. These two immunoassays have, respectively, allowed the detection of 6 p 1000 and 5 ppm bovine crude heparin in porcine heparin [Rivera et al., J. Pharm. Biomed. Anal, submitted; Levieux et al., J. Immunoassay, submitted].

In this work, in order to fully characterise Ag1, we firstly determined its histological localisation in bovine small intestine cryosections. Then, we studied its stability to heat and pH treatments. Finally, using chromatographic techniques and SDS-PAGE, Ag1 was isolated, partly sequenced and identified as an aprotinin/heparin complex.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), human serum albumin (HSA), *o*-phenylenediamine, heparinase I (EC 4.2.2.7), bovine mucosal pure heparin, Triton X100, aprotinin and bovine trypsin were purchased from Sigma Chemical Company (St Louis, MO, USA). Horseradish-peroxidase and rhodamine labelled goat anti-rabbit immunoglobulins were from Nordic Immunology (Tebu, Le Perray-en-Yvelines, France). Paraformaldehyde and sucrose were supplied by Prolabo (Fontenay-sur-bois, France). Skimmed milk powder was purchased from Regilait (Saint-Martin-Belle-Roche, France) and Tween 20 from Merck (Hohenbrunn, Germany). BCA protein assay reagent and Immunopure® Metal Enhanced DAB Substrate Kit were supplied by Pierce (Rockford, IL, USA). Bovine crude heparin was extracted from hashed gut using macroporous anion-exchange resin as previously described [Rivera et al., J. Pharm. Biomed. Anal., submitted]. All other chemicals were of analytical grade.

2.2. Antisera production

Anti-Ag1 antiserum was produced in rabbits by multiple intradermal injections of bovine crude heparin (50 mg/ml) at monthly interval as previously described [Rivera et al., J. Pharm. Biomed. Anal., submitted]. Contaminating antibodies against BSA were removed by immunoabsorption with BSA beads obtained by glutaraldehyde polymerisation [14]. The specificity of the antiserum and the efficiency of the absorption were controlled by double immunodiffusion and indirect ELISA using a coating of 100 ng/ml BSA.

Anti-bovine trypsin antiserum was similarly obtained in rabbits using 1 mg/ml bovine trypsin as immunogen.

2.3. Immunohistochemical localisation of Ag1 in bovine small intestine cryosections

Fresh bovine small intestine collected at the slaughterhouse of the research centre was emptied of its faecal content and cut into strips of approximately 1 cm width. The strips were delicately washed in phosphate buffered saline (pH 7.4; 0.01 M) (PBS), fixed in 1.5% paraformaldehyde in PBS (v/v) for 90 min at 4 °C and gradually infiltrated with PBS–sucrose solutions, upto a final concentration of 30% sucrose (w/v). Sections (8 μm thickness) were cut at −25 °C, mounted on Superfrost Plus microscope slides (GmbH and Co) and dried overnight at room temperature.

For immunostaining, non-specific binding sites were firstly blocked by incubation of the sections for 1 h with 3% HSA in PBS (w/v). The sections were then washed for 3 min in PBS and incubated for 1 h with anti-Ag1 antiserum diluted 1/300 (v/v). After washing three times for 5 min in PBS, the sections were incubated with a rhodamine-labelled goat anti-rabbit immunoglobulin G diluted 1/300 (v/v). The sections were then rinsed in PBS containing 0.1% Tween 20 (v/v) (3 × 5 min) and viewed under an epifluorescent microscope. Specific controls were performed by substituting the primary antiserum for a non-immune rabbit serum at the same dilution. PBS containing 0.3% HSA (w/v) and 0.1% Tween 20 (v/v) was used as diluent for the antisera.

To localise the specific areas detected by immunostaining, light microscopic observations were made on alternate sections stained as follows: incubation in Harris Haematoxylin for 5 min, incubation in 0.5% Eosin Y (w/v) for 15 s, dehydration in ethanol solutions (80, 95% and absolute ethanol, respectively) and clearance in methyl-cyclohexan.

2.4. Agar gel immunoprecipitation techniques

Agar gel double immunodiffusion, immunoelectrophoresis, fused-rocket and line immunoelectrophoresis were performed as previously described [Rivera et al., J. Pharm. Biomed. Anal., submitted].

2.5. Quantification of Ag1

The concentration of Ag1 in tissue extracts and chromatographic fractions was determined by SRID as previously described [Rivera et al., J. Pharm. Biomed. Anal., submitted]. Ag1 concentration in chromatographic fractions was alternatively determined by immunonephelometry using the anti-Ag1 antiserum. The rate nephelometer (ImmunoChemistry System 'ICS', quartz iodine lamp 400–500 nm, scatter angle 70°; Beckman Instruments, Gagny, France) was operated in the manual mode using the M33 sensitivity.

2.6. Heat stability studies

Aliquots (400 μl) of a bovine crude heparin solution (10 mg/ml in PBS) in evacuated, sealed glass tubes were heated between 60 and 80 °C in a thermostatically controlled waterbath (Polystat 44, Bioblock Scientific, Illkirch, France) or in boiling water (97 °C) for 30 min. Heat treatment was halted by immersion in ice water. An unheated aliquot was used as a control. Samples were centrifuged at 10 000 × g for 5 min and 0.2 μm filtered. The Ag1 concentration was determined by SRID.

2.7. pH stability studies

The pH effect was examined in the range 2–12 (0.1 M buffer stock solutions of glycine–HCl for pH 2, sodium citrate for pH 3 and 4, sodium acetate for pH 5, Bis–Tris–HCl for pH 6 and 7, Tris–HCl for pH 8, ethanolamine for pH 9, glycine–NaOH for pH 10 and 11, and disodium phosphate for pH 12. Bovine crude heparin (10 mg/ml final concentration) was incubated at room temperature at each pH for 1 h and the residual Ag1 reactivity was determined at pH 7.0–7.5 by SRID.

2.8. Preparation of tissue extracts

Bovine small intestine and lung were freshly obtained from the slaughterhouse of the research centre. Small intestine was emptied of its faecal content, cut into small pieces and chopped in a robot coupe commercial processor (Moulinex, France) in order to obtain hashed gut. Hashed muscular layers plus serous membranes (hashed membranes) were obtained as described for hashed gut but, in this case, intestinal mucosa was scraped off before chopping. Hashed lung was processed as described for the hashed gut. A typical extraction consisted of adding 10 ml of Tris–HCl buffer (pH 7.5; 0.1 M) 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 $25\,000 \times g$ for 15 min at 4 °C and the supernatant was collected.

2.9. Precipitation of tissues extracts by ammonium sulphate or octanoic acid

A saturated solution of ammonium sulphate (4.1 M) equilibrated at 4 °C was added dropwise (33–66% final concentration) under continuous stirring of hashed membranes extracts immersed in ice water. After mixing for 5 min, samples were centrifuged at $25\,000 \times g$ for 25 min at 4 °C and the supernatant 0.45 μm filtered.

For octanoic acid precipitation, the pH of the hashed membranes extract was first adjusted to 4.5 with 0.5 M HCl. Then, octanoic acid (10–40 μl) was added dropwise to 1 ml aliquots of hashed membranes extracts under continuous stirring. Samples were then treated as described for ammonium sulphate precipitation. An untreated aliquot adjusted to pH 4.5 was used as a control.

Ag1 concentration in the supernatants was determined by SRID. Protein concentration was determined with the BCA Protein Assay Reagent (Pierce) using BSA as standard.

2.10. Purification of Ag1 from bovine crude heparin by ion-exchange and size-exclusion High Pressure Liquid Chromatography (HPLC)

Bovine crude heparin (1 ml at 20 mg/ml) in sodium phosphate buffer (pH 7.0; 0.1 M) was injected in a Mono Q anion-exchange column (HR 10/10, Pharmacia Biotech, S-751 82 Uppsala, Sweden) previously equilibrated with the same buffer. Elution was performed at 1 ml/min using a HPLC system (Pump 420, Detector 430; Kontron Instruments, F-78180 Montigny-les-Bretonneux, France) in sodium phosphate buffer (pH 7.0; 0.1 M) using a three steps NaCl gradient: 0–0.8 M (7 min), 0.8–1.6 M (20 min) and 1.6–2.0 M (3 min). Fractions showing maximum reactivity in light scattering measurements were pooled, 27-fold concentrated by ultrafiltration (10 kDa cut-off) and loaded onto a 21×25 mm Zorbax GF250 XL column (Dupont de Nemours, Wilmington, DE, USA) previously equilibrated with sodium phosphate buffer (pH 7.0; 0.1 M; 1 M NaCl). Elution was performed with the equilibration buffer at 5 ml/min using the HPLC equipment. Ag1 was monitored in the fractions by immunonephelometry. Bovine whey supplemented with 3 mg/ml of BSA was used for the column calibration: the molecular weight of immunoglobulin G (IgG, 160 kDa), BSA (67 kDa), β-lactoglobulin (β-lg, 36.6 kDa) and α-lactalbumin (α-la, 14.4 kDa) were plotted versus their retention time.

2.11. Purification of Ag1 from bovine intestine by size-exclusion and ion-exchange chromatography

An extract of hashed membranes was precipitated by addition of saturated ammonium sulphate to a final concentration of 40% (v/v) as

described above. After centrifugation at 25 000 × *g* for 25 min, the supernatant was dialysed (12–14 kDa cut-off) overnight against Tris–HCl buffer (pH 8.5; 0.05 M) at 4 °C, 21-fold concentrated by ultrafiltration (10 kDa cut-off) and loaded onto a 850 × 25 mm Sephadex G100 Superfine column (Pharmacia Biotech) previously equilibrated with the same buffer. Elution was performed at 4 °C with the equilibration buffer at a flow rate of 13 ml/h**.** For the calibration of the column, the following standards were used: IgG, BSA, ovalbumin (OVA, 43 kDa) and cytochrome C (Cyt.C, 12.4 kDa). Fractions with maximum Ag1 immunoreactivity were pooled and loaded onto the Mono Q anion-exchange column previously equilibrated with Tris–HCl buffer (pH 8.5; 0.05 M). Elution was performed at 2 ml/min using the HPLC system with a four steps NaCl gradient: 0–0.2 M (17 min), 0.2–0.9 M (6 min) and 0.9–1.5 M (6 min) after 4 min at 0.9 M. The fractions with maximum Ag1 immunoreactivity obtained from two runs were pooled and dialysed against the equilibration buffer. The pool was loaded again onto the Mono Q anion-exchange column and concentrated by reverse elution of the column at 1 ml/min with Tris–HCl buffer (pH 8.5; 0.05 M; 0.15 M NaCl). Ag1 concentration in the fractions was monitored by SRID.

2.12. Purification of Ag1 from bovine crude heparin or hashed membranes by immuno-affinity chromatography

The IgG fraction of the anti-Ag1 antiserum was purified by anion-exchange chromatography on a Q Sepharose Fast-Flow column (Amersham Pharmacia Biotech) using a linear NaCl gradient (0–0.5 M) in Tris–HCl buffer (pH 8.5; 0.02 M) with monitoring the absorbance at 280 nm and 405 nm. The IgG fraction (5 ml, 2.9 mg/ml) was then dialysed at 4 °C against sodium carbonate buffer (pH 8.3; 0.1 M; 0.5 M NaCl) and coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). The immunosorbent was then poured into an 11 × 70 mm IBF 11G column (IBF Biotechnics, France). The settled bed volume of the packed gel was 2.4 ml.

Bovine crude heparin (10 mg/ml) was loaded at 13 ml/h on the affinity column previously equilibrated with Tris–HCl buffer (pH 8.2; 0.1 M; 0.5 M NaCl). The column was then washed with 7 column volumes of the equilibration buffer and developed glycine–HCl buffer (pH 2.0; 0.1 M; 1 M NaCl) while monitoring the absorbance at 280 nm. The eluted fractions were immediately neutralised with Tris–HCl buffer (pH 8.0; 1 M) and monitored for Ag1 concentration by SRID. Fractions containing Ag1 were pooled, exhaustively dialysed (12–14 kDa cut-off) against Tris–HCl buffer (pH 8.5; 0.05 M) and stored at 4 °C.

An extract of hashed membranes precipitated with 40% ammonium sulphate as described above was desalted by gel permeation on a Bio-Gel P-6DG column (Biorad) previously equilibrated with Tris–HCl buffer (pH 8.2; 0.1 M; 0.5 M NaCl). The sample (12 ml) was purified on the affinity column as described above for bovine crude heparin. As a final step, the eluted and neutralised fractions were exhaustively dialysed against Ultrapure Milli Q water and finally lyophilised. All experiments were done at 4 °C.

2.13. Enzymatic depolymerisation of bovine crude heparin

Heparinase I was dissolved (0.5 IU/ml) in potassium phosphate buffer (pH 7.0; 10 mM) containing 40 μg/ml BSA, aliquoted and stored at −20 °C before use. Bovine crude heparin was dissolved (20 mg/ml) in calcium acetate buffer (pH 7.0; 2 mM) containing 100 μg/ml BSA. Hydrolysis was performed at 25 °C by mixing 0.64 ml of bovine crude heparin solution, 2.24 ml of the calcium acetate buffer and 0.32 ml of heparinase solution in stoppered glass tubes. A negative control was made by omitting heparinase in the potassium phosphate buffer. After 48 h running, samples were exhaustively dialysed at 4 °C against Ultrapure Milli Q water (6–8 kDa cut-off), 0.2 μm filtered and lyophilised.

2.14. Analysis of heparinase digest by size-exclusion HPLC

The lyophilised heparinase digest (1 mg) was

dissolved in 0.2 ml Tris–HCl buffer (pH 8.0; 0.1 M; 2 M NaCl) and loaded onto a 9.4 × 25 mm Zorbax GF250 column previously equilibrated with the same buffer. Elution was performed at 1 ml/min with the equilibration buffer by using the HPLC equipment. Ag1 concentration in fractions was monitored by SRID.

2.15. SDS-PAGE analyses

Samples were diluted (v/v) with Tris–HCl buffer (pH 6.8; 0.05 M) containing 2.5% SDS, 7.5% glycerol and heat denatured in boiling water for 8 min. They were subjected to SDS-PAGE according to Laemmli [15] using 10 or 12.5% acrylamide slab gels. Gels were stained by Coomassie brilliant blue R250 or silver nitrate. Molecular weight standards (Amersham Pharmacia Biotech) contained phosphorylase B (94.0 kDa), BSA (67.0 kDa), OVA (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-la (14.4 kDa).

2.16. Immunoblotting

After SDS-PAGE, proteins were transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech) by semi-dry blotting (15 V for 15 min). Standard proteins were located by reversible staining with Ponceau red (0.2% in 1% trichloroacetic acid). Non-specific binding sites were blocked by soaking the membrane in PBS containing 10% skimmed dry milk (w/v) for 1 h. The membrane was then incubated for 1 h with 1/1000 (v/v) anti-Ag1 antiserum. After washing (4 × 5 min), the membrane was incubated for 1 h in the dark with a peroxidase-labelled goat anti-rabbit IgG diluted 1/1500 (v/v). After four washes in the dark the membrane was rinsed with PBS and immunocomplexes were revealed using the Immunopure® Metal Enhanced DAB Substrate Kit. Controls were performed by substituting the primary antiserum for a non-immune rabbit serum diluted 1/1000 (v/v). PBS containing 1% dry milk (w/v) and 0.1% Triton X 100 (v/v) was used as diluent and washing solution.

2.17. Electrophoresis in agarose

Electrophoresis in agarose gels was performed using the Hydragel-Mini HR Kit (Sebia) with the Sebia K20 chamber according to manufacturer's instructions. The sample application template was placed onto the cathodic side of the gel instead of in the centre. After electrophoresis, gels were fixed for 10 min in an ethanol–water–acetic acid solution (30:65:5, v/v/v), air-dried and then stained for 30 min with Toluidine blue (1 g/l in fixation solution). Destaining was performed in the fixation solution until the background was completely colourless and clear. For Western blotting analysis with the anti-Ag1 antiserum, samples were transferred from an unfixed gel to a PVDF membrane by pressing for 15 s. All subsequent steps were performed as described above in the immunoblotting section.

2.18. Amino acid sequencing of Ag1

After SDS-PAGE and Coomassie staining/destaining, the acrylamide gel was extensively washed with Ultrapure Milli Q water and the 16 kDa band excised. Internal peptides were obtained by in situ digestion with 0.1 μg trypsin in 200 μl Tris–HCl buffer (Ph 8.6; 0.05 M) containing 0.01% Tween 20 (w/v) for 18 h at 30 °C. Peptides were purified by HPLC on successive Aquapore AX-300 (DEAE, 7μ, 2.1 mm i.d. × 30 mm, Applied Biosystem) and Vydac 218TP52 (C18, 300 Å, 2.1 mm i.d. × 250 mm) columns with a gradient of 2–70% acetonitrile in 0.2% trifluoroacetic acid at a flow rate of 200 μl/min. The amino acid sequences were determined by the Dansyl–Edman technique using an Applied Biosystems Procise Sequencer. Sequences were checked for homology in the Swiss–Prot database by the Mail-FASTA service using the Blast program.

3. Results

3.1. Specificity of the anti-Ag1 antiserum

The efficiency of the immunoabsorption of the

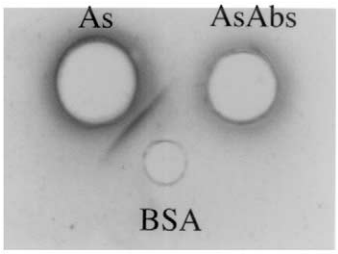

Fig. 1. Agar gel double immunodiffusion analysis of the anti-Ag1 antiserum before (As) and after (AsAbs) immunoabsorption on BSA beads. Lower hole:BSA.

contaminating anti-BSA antibodies was firstly controlled by agar gel double immunodiffusion (Fig. 1). However, small quantities of residual anti-BSA antibodies could have given false positive results in Western blotting and immunohistological analyses. Thus, the immunoabsorbed anti-Ag1 antiserum was more sensitively controlled by indirect ELISA. No significant optical density was observed at the working dilution 1/300–1/1000 (data not shown).

3.2. Immunohistochemical localisation of Ag1 in bovine small intestine cryosections

Using the absorbed antiserum, the immunohistochemical localisation of Ag1 in bovine small intestine cryosections was investigated. In preliminary experiments, several procedures for fixation (0.5–1.5% glutaraldehyde, 1–3% paraformaldehyde) and freezing (direct freezing of the tissue in isopentan at −180 °C, freezing in the cryostat using sucrose as cryoprotector) were evaluated. Immunohistochemical staining of the sections was also tested by using different blocking agents (normal goat serum, goat IgG, HSA, BSA) and 2 fluorescent conjugates (FITC and rhodamine goat anti-rabbit immunoglobulins). Finally, a satisfactory preservation of tissue morphology without loss of Ag1 immunoreactivity was found using 1.5% paraformaldehyde as fixative, infiltration of the tissue with 30% sucrose and freezing at −25 °C. Saturation of the sections with 3% HSA (w/v) and revelation of immunocomplexes with rhodamine-conjugate gave optimal results. Using this procedure, high-intensity fluorescent labelling was found in the serous membrane and at the junction between this membrane and the external longitudinal smooth muscular layer (Fig. 2a). Only very low fluorescent background was detected upon substitution of the absorbed anti-Ag1 antiserum for a normal rabbit serum at the same dilution (Fig. 2b).

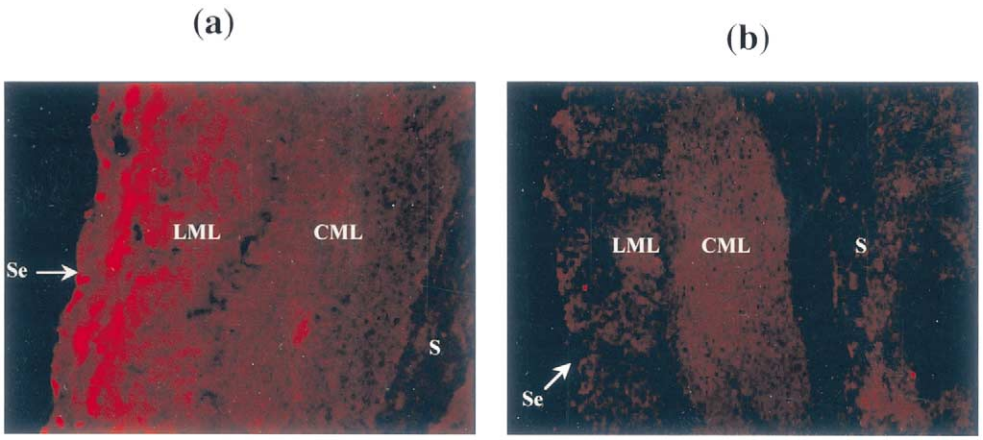

Fig. 2. Localisation of Ag1 in bovine small intestine cryosection by immunostaining with a fluorescent rhodamine conjugate (see Section 2). (a) Specific immunostaining with the rabbit anti-Ag1 antiserum (× 200). (b) Control experiment performed by substituting the specific anti-Ag1 antiserum for a normal rabbit serum at the same dilution (× 200). Se, Serous membrane; LML, external Longitudinal smooth Muscular Layer; CML, external Circular smooth Muscular Layer; S, Sub-mucosa.

3.3. Heat and pH stability of Ag1 in bovine crude heparin

Heating of bovine crude heparin solutions showed that Ag1 remained unaltered upto 80 °C for 30 min. After boiling for the same time, 67% of native Ag1 was still found.

When Ag1 was kept for 1 h at different pHs ranging from 2 to 12, no immunoreactivity losses were noted, suggesting a high pH stability of Ag1.

3.4. Purification of Ag1 from bovine crude heparin or bovine intestine by ion-exchange and size-exclusion chromatography

The elution profile of a bovine crude heparin chromatographied on the anion-exchange Mono Q HR 10/10 column is shown in Fig. 3a. Ag1 was found in the fractions eluted in the range 1.1–1.3 M NaCl, with a maximum at 1.20 M NaCl concentration. The immunoreactive fractions were pooled, concentrated and injected onto a Zorbax GF250 size-exclusion column. Ag1 displayed a molecular weight distribution from 15 to 300 kDa (Fig. 3b). The average molecular weight of Ag1 was 45 kDa, result in agreement with previously reported data (Rivera et al., J. Pharm. Biomed Anal., submitted). Attempts to check the purity of this 45 kDa fraction by SDS-PAGE and Western blotting were unsuccessful since large smears were obtained, presumably because of the high concentration of heparin in the sample (data not shown).

Since the purification of Ag1 could not be easily monitored using bovine crude heparin as starting material, the feasibility of its purification from bovine intestine was investigated. Beforehand, the characterisation of Ag1 in tissue extracts was performed by agarose-gel immunoprecipitation, SDS-PAGE and Western blotting.

By immunoelectrophoresis, Ag1 was found in a less anodic position for a hashed gut extract than for bovine crude heparin (Fig. 4a). By fused-rocket analysis, Ag1 migrated exclusively toward the anode in crude heparin, but in the hashed gut extract the antigen was unexpectedly found in both anodic and cathodic position (Fig. 4b). By agar gel double immunodiffusion, a complete

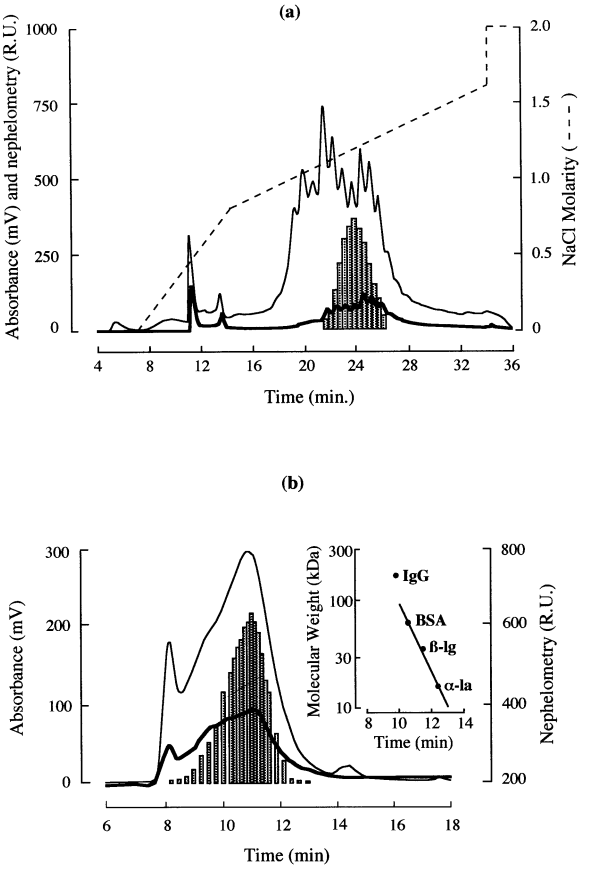

Fig. 3. HPLC purification of Ag1 from bovine crude heparin (20 mg/ml) by (a) anion-exchange on a Mono Q HR 10/10 column followed by (b) size-exclusion on a Zorbax GF250 XL column. The column calibration (inset) was performed with bovine whey supplemented with BSA. A_{280} (—); A_{214} (—); NaCl molar concentration (---). Ag1 immunoreactivity (histogram) was monitored in the fractions by immunonephelometry and was expressed in Rate Units (R.U.).

antigenic identity was found for Ag1 between bovine small intestine and lung (Fig. 4c). Since the latter organ was found to be three times more concentrated in Ag1 than small intestine, the dual electrophoretic migration of Ag1 was more clearly revealed by line-immunoelectrophoresis analyses in the case of a hashed lung extract, the cathodic migration being largely prevalent (Fig. 4d).

The profiles obtained by SDS-PAGE analysis of hashed gut and lung extracts were roughly

similar with three major common bands at 67, 43, and around 17 kDa, and two additional major bands at 57 and 30 kDa for hashed lung (Fig. 5a). Western blotting analyses using the anti-Ag1 antiserum, performed after SDS-PAGE in reducing and non reducing conditions, enabled the determination of an apparent molecular weight of 30 kDa for Ag1 in both tissular extracts. In the hashed gut, another thin minor band with an apparent molecular weight of 28 kDa was also revealed (Fig. 5b). In subsequent Western blotting analysis, a faint band near the front was frequently observed (Fig. 8a, Fig. 9c). Similar patterns were observed for hashed membranes extract (data not shown). However, the ratio Ag1/protein content was found twice as high in hashed membranes as in hashed gut extract. Consequently, hashed membranes were selected for Ag1 purification from gut.

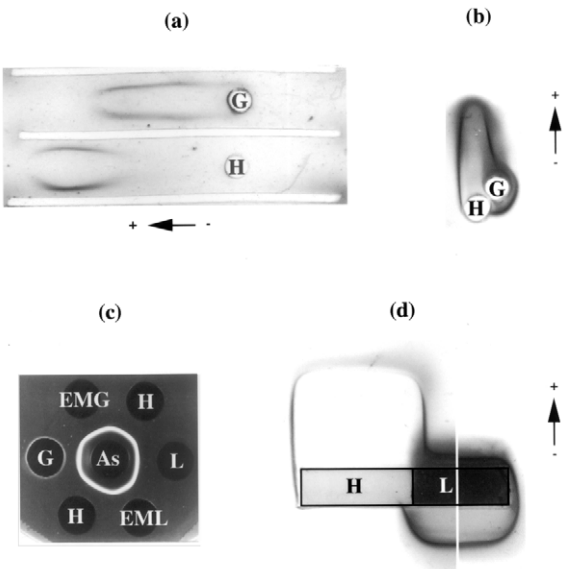

Fig. 4. Characterisation of Ag1 in tissue extracts by agar gel immunoprecipitation techniques using the anti-Ag1 antiserum. (a) Immunoelectrophoresis of bovine hashed gut extract (G) and bovine crude heparin (H) by using the anti-Ag1 antiserum (troughs). (b) Fused rocket immunoelectrophoresis of bovine crude heparin (H) and bovine hashed gut extract (G). (c) Analyses of bovine samples by agar gel double immunodiffusion. H, bovine crude heparin (control); G, hashed gut extract; L, hashed lung extract; EMG and EML, Eluted Material issued from the chromatographic steps of bovine Gut and bovine Lung heparin preparations, respectively; As, anti-Ag1 antiserum. (d) Line immunoelectrophoresis of bovine crude heparin (H) and hashed lung extract (L).

Optimal conditions for the elimination of residual proteins by precipitation with ammonium sulphate or octanoic acid was defined (Table 1). The best yield was obtained using ammonium sulphate to a final concentration of 40%. In this condition, 83% of Ag1 content were recovered whereas 75% of the protein content were eliminated.

The elution profile of a hashed membranes extract precipitated by 40% of ammonium sulphate and chromatographied on a Sephadex G100 superfine column is shown in Fig. 6a. Ag1 immunoreactivity was recovered in the major peak with an average apparent molecular weight of 69 kDa. However, only 65% of the Ag1 loaded on the column were recovered after this chromatographic step. The immunoreactive fractions were pooled and loaded onto a Mono Q HR 10/10 column. Ag1 was detected essentially in the range 0.02–0.12 M NaCl, but also a minor reactivity was found in the unbound fractions and in the 1.20 M NaCl fraction (Fig. 6b)

The SDS-PAGE of the concentrated 0.02–0.12 M NaCl fractions revealed the presence of a major band at the expected apparent molecular weight of 69 kDa and another one at low molecular weight (< 20 kDa) (Fig. 7a). Furthermore, the immunoelectrophoretic analysis of this pool showed a unique arc with an anodic electrophoretic mobility lower than that observed for Ag1 in bovine crude heparin (Fig. 7b).

Amino acid sequencing of 2 peptidic fragments obtained after enzymatic proteolysis of the 69 kDa protein band revealed a 100% homology with bovine transferrin, a glycoprotein essentially found in the serum. However, owing to the higher resistance of Ag1 to heat treatment and to its absence in bovine serum and muscle (Rivera et al., J. Pharm. Biomed. Anal., submitted) we concluded that this 69 kDa protein was not Ag1 but a contaminant co-purified with Ag1. This assumption was subsequently confirmed in Western blotting by the recovery of the major immunoreactivity in the low molecular weight band (data not shown).

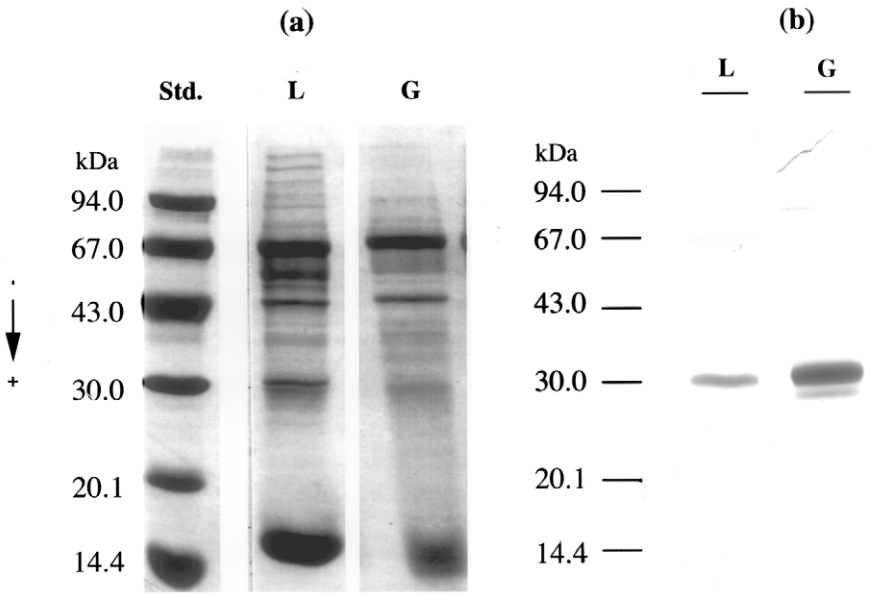

Fig. 5. Analyses of hashed lung and hashed gut extracts by SDS-PAGE and Western blotting. (a) SDS-PAGE obtained after Coomassie staining on 12% gel. Std, molecular weight markers; L, hashed lung extract; G, hashed gut extract. (b) Western blotting analysis on PVDF membrane revealed using the anti-Ag1 antiserum.

3.5. Purification of Ag1 from bovine crude heparin or intestinal extract by immuno-affinity chromatography

Since the combination of size-exclusion and ion-exchange chromatographies gave unsatisfactory results, we attempted to purify Ag1 using affinity chromatography. For this purpose, the IgG of the anti-Ag1 antiserum were purified by anion-exchange chromatography and coupled to CNBr activated Sepharose 4B. Then, the immunoadsorbent was used to purify Ag1 from bovine crude heparin. More than 90% of the immunoreactive Ag1 loaded on the column was recovered in the fraction eluted with acidic buffer. No bands were detected by SDS-PAGE of this fraction after Coomassie or silver staining. However, after carbohydrate staining with Toluidine blue (data not shown) or Western blotting by using the anti-Ag1 antiserum (Fig. 8a), a smear extending approximately from 30 to 50 kDa was detected. In the Western blotting analyses, an additional faint band was revealed at low molecular weight (< 14 kDa). On electrophoresis in Hydragel agarose, a thin band and a smear were revealed both after Toluidine blue staining (Fig. 8b) and blotting of the gel on a PVDF membrane and immunostaining with the anti-Ag1 antiserum (Fig. 8c).

Table 1
Recovery of Ag1 in a hashed membranes extract after protein precipitation with varying proportions of ammonium sulphate or octanoic acid

Precipitating agent	Residual concentration (%)	
	Ag1	Protein
Ammonium sulfate (%)		
33	98	39
40	83	25
50	53	15
60	27	7
66	0	3
Octanoic acid		
10	85	75
20	81	72
30	56	63
40	46	57

Values are means of duplicates.

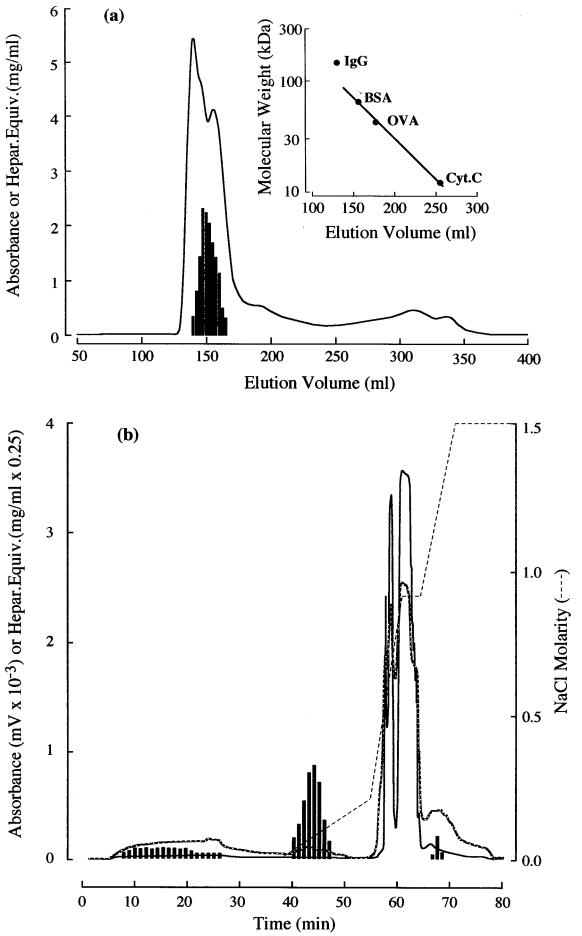

Fig. 6. Purification of Ag1 from a bovine hashed membrane extract ammonium sulphate precipitated by (a) size-exclusion chromatography on Shephadex G100 Superfine column followed by (b) anion-exchange HLPC on Mono Q HR 10/10 column. Ag1 concentration in the fractions (histogram) was monitored by SRID. Results were expressed as the equivalent concentration of bovine crude heparin (Hepar.Equiv. (mg/ml)) which gave the same reactivity as Ag1 present in the fractions. The calibration of the Shephadex G100 Superfine column with proteins of known molecular weight is inset on (a). A_{280} (—); A_{214} (—); NaCl molar concentration (---).

The anti-Ag1 immunoadsorbent was also used to purify Ag1 from hashed membranes extract treated with 40% ammonium sulphate. The desalting step on the Bio-Gel P-6DG column lead to a loss of 50% of Ag1. By affinity chromatography, the Ag1 loaded onto the column was fully recovered in the fraction eluted at pH 2.0 (Fig. 9a). SDS-PAGE of this fraction revealed two bands with apparent molecular weights of 14 and 16 kDa (Fig. 9b). Western blot analysis of the gel revealed the presence of a strong band with an apparent molecular weight of 16 kDa, a faint band at a molecular weight inferior to 14.4 kDa and a smear in the 20–30 kDa zone (Fig. 9c).

Tryptic digestion of the 16 kDa protein gave a particularly low peptide yield. The amino acid sequence obtained for the most abundant peptide was RPDFXLEPPYTGPX. Considering the X residues as cystein, a 100% homology with the N-terminal sequence of bovine pancreatic trypsin inhibitor (BPTI), also commonly called aprotinin, was found.

This result was particularly surprising since aprotinin has been described as a strongly basic protein (pI 10.5) with a low molecular weight (6.5 kDa) [16]. Nevertheless, a complete identity between Ag1 and commercial aprotinin was confirmed by agar gel double immunodiffusion (Fig. 10a).

However, the discordance between the molecular weight of aprotinin and Ag1 in crude heparin together with the detection of smears in SDS-

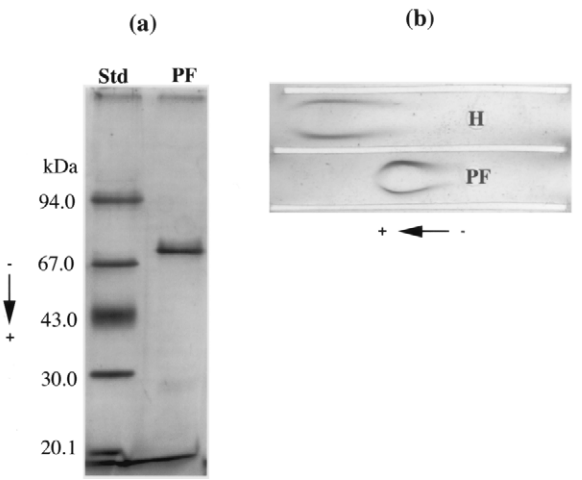

Fig. 7. SDS-PAGE and immunoelectrophoresis of the fraction obtained from a bovine hashed membranes extract after size-exclusion and anion-exchange HPLC chromatography. (a) 10% SDS-PAGE with silver staining. Std, standards proteins; PF, purified fraction. (b) Immunoelectrophoresis of the purified fraction (PF). H, bovine crude heparin (control); troughs: anti-Ag1 antiserum.

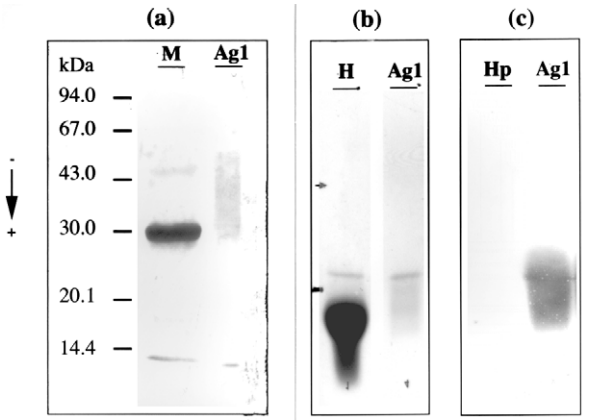

Fig. 8. Analyses of the fraction obtained from bovine crude heparin by immunoaffinity chromatography. (a) Western blotting analyses after transfer of a 12% SDS-PAGE to a PVDF membrane. M, hashed membranes extract (positive control); Ag1, fraction eluted from the affinity column. (b) Electrophoresis on Hydragel agarose with Toluidine blue staining. H, bovine crude heparin (reference). (c) Immunoanalysis of the blotting obtained after transfer from the Hydragel agarose to a PVDF membrane. Hp, bovine pure heparin. (a) and (c) were revealed by using the anti-Ag1 antiserum.

PAGE and Western blot analyses of bovine crude heparin (Fig. 8), allowed us to make the assumption that Ag1 in crude heparin was a complex of aprotinin bound to heparin chains. The digestion of heparin in bovine crude heparin with heparinase I confirmed this hypothesis since a marked decrease in the immunoelectrophoretic mobility (Fig. 10b) as well as in molecular weight (Fig. 10c) was observed for Ag1. However, according to apparent molecular weight of 10.5 kDa and the wide arc observed in immunoelectrophoresis, we concluded that heparin fragments were still bound to aprotinin after hydrolysis.

4. Discussion

Owing to the potential risk of contamination with the BSE agent using drugs derived from ruminant materials, heparin purification is currently restricted to porcine intestinal mucosa in western countries. Since the physico-chemical methods developed for the control of pure heparin batches are not sensitive enough, the development of complementary methods to control the animal origin of the intermediate crude products is required to ensure the safety of the drug. In this context, an immunochemical approach intended to detect residual contaminants in intestinal bovine crude heparin has been recently investigated [Rivera et al., J. Pharm. Biomed. Anal., submitted]. The finding of a strictly bovine-specific antigen, called Ag1, has opened the possibility to control crude heparin batches with regard to bovine contamination, and a very sensitive ELISA has been developed [Levieux et al., J. Immunoassay, submitted]. However, for a better knowledge of the possibilities and limits of the immunochemical control of crude heparins, a detailed characterisation of Ag1 was needed. We, thus, studied here the histological localisation and stability to heat and pH of Ag1 and we attempted to purify this antigen using different chromatographic approaches and sources. Sequencing of an internal peptide from a purified fraction revealed a complete homology with aprotinin, a protein also known as the BPTI. This result was confirmed by the finding of complete immunochemical identity between Ag1 and aprotinin using double immunodiffusion experiment. However, Ag1 is found in heparin as a complex between heparin chains and aprotinin as evidenced by the decrease both in molecular weight and electrophoretic mobility observed after heparinase digestion of bovine crude heparin.

The immunohistological localisation of Ag1 in the serous membrane and at the junction between this membrane and the external longitudinal smooth muscular layer is in accordance with current knowledge about aprotinin. This inhibitor has been immuno-localised in mast cells [17–20], and, thus, found in virtually all bovine organs [16,21–23] and particularly in connective tissues. Moreover, as already reported by Fritz et al. [17] the preparation of tissue sections for immunohistological studies turned out to be a special problem since the rupture of the mast cell granules integrity led to aprotinin diffusion and consequently to unspecific staining. We only obtained satisfactory results when the sections were fixed with 1.5% paraformaldehyde, impregnated with 30% sucrose and slowly freezed at -25 °C.

Ag1 was found to be particularly resistant to heat treatment since only 33% of its immunological reactivity were lost after 30 min boiling. Moreover, its immunological reactivity was not modified by 1 h standing at pHs ranging from 2 to 12. These results are consistent with the description of aprotinin as a small globular protein of 6.5 kDa consisting in a single polypeptide chain of 58 amino acid residues stabilised by three disulphide bonds [16]. This arrangement results in a very compact tertiary structure which explain its high stability toward thermic and chemical denaturation as well as its resistance to proteolytic digestion [24,25].

The variety of molecular weight observed for Ag1 when crude heparin or tissular extracts were chromatographied, as well as the smears revealed by carbohydrate staining and Western blotting can be easily explained by the capacity of aprotinin to bind to heparin [26]. This binding in

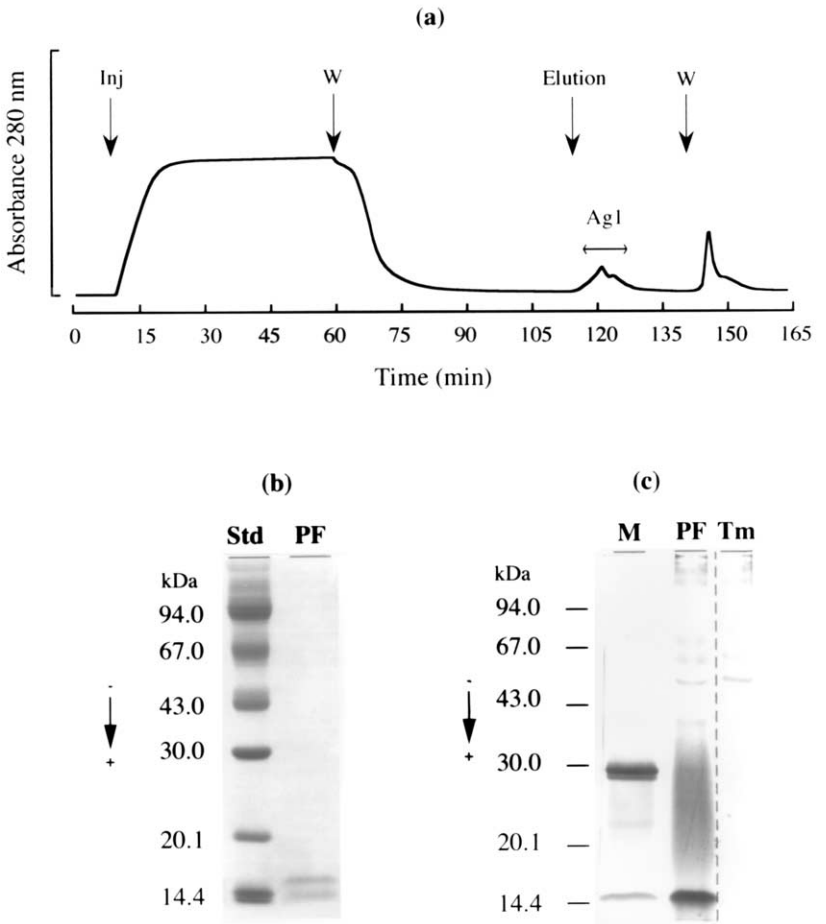

Fig. 9. Purification of Ag1 from a bovine hashed membranes extract by immunoaffinity chromatography and analyses of the eluted fraction by SDS-PAGE and Western blotting. (a) A bovine hashed membranes extract precipitated with 40% ammonium sulphate solution was injected onto the affinity column (Inj). The column was washed with Tris-HCl buffer (pH 8.2; 0.1 M; 0.5 M NaCl) (W) and elution was performed with glycine-HCl buffer (pH 2.0; 0.1 M; 1 M NaCl) (Elution). Ag1 was monitored in the fractions by SRID (↔). A_{280} (—). (b) 12% SDS-PAGE obtained for the purified fraction (PF) with Coomassie staining. Std, molecular weight markers. (c). Western blotting of the PF on a PVDF membrane using the anti-Ag1 antiserum. M, hashed membranes extract precipitated with ammonium sulphate (positive control); Tm, analyses of the PF track by substituting anti-Ag1 antiserum for a normal rabbit serum.

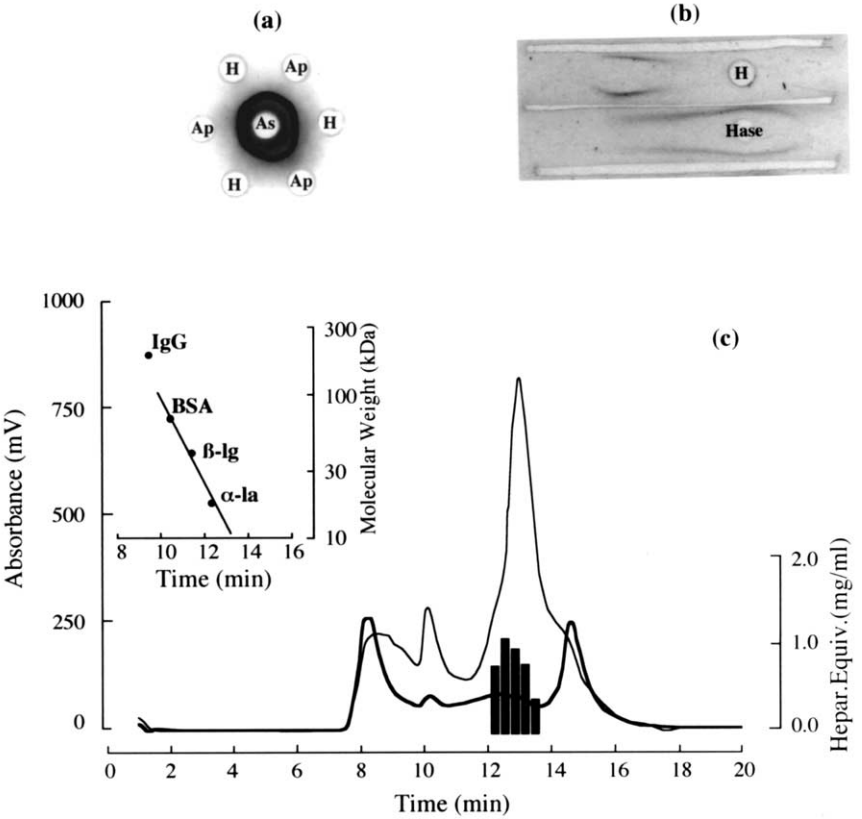

Fig. 10. Identification of Ag1 as an aprotinin/heparin complex. (a) Double immunodiffusion analysis of bovine crude heparin (H) and commercial aprotinin (Ap) at different concentration with the anti-Ag1 antiserum (As). (b) Immunoelectrophoretic analyses of bovine crude heparin before (H) and after (Hase) heparinase I digestion. Troughs: anti-Ag1 antiserum. (c) Elution profile of Hase from the Zorbax GF250 size-exclusion column. Elution was performed at 1 ml/min with Tris–HCl buffer (pH 8.0; 0.1 M; 2 M NaCl). Ag1 was monitored in the fractions by SRID and was expressed in Hepar.Equiv. (mg/ml) as in Fig. 6. Inset: column calibration. A_{280} (—); A_{214} (—); Histogram: Ag1 immunoreactivity.

bovine crude heparin was confirmed by the significant decrease in both molecular weight and anodic electrophoretic migration of Ag1 after heparinase I digestion. Aprotinin binds also to a variety of enzymes belonging to the serine protease group, forming tight complexes [16]. Of special interest, the aprotinin-trypsin complex has been found to be extremely stable ($Kd = 6 \times 10^{-14}$ M) [27] and resistant to dissociation by boiling with SDS [28]. Accordingly, it could be assumed that the 30 kDa band revealed by Western blotting analyses of intestinal extract was an equimolar complex between trypsin (24 kDa) and aprotinin (6.5 kDa). However, this assumption was not confirmed since no bands were revealed both at 24 and 30 kDa using an anti-bovine trypsin antiserum in Western blotting analyses. Binding of aprotinin to glycoproteins has also been described [26]. This could explain the co-purification of transferrin with aprotinin and the immunological reactivity found around 70 kDa during gel permeation on Sephadex G100. Owing to the heat sensitivity of bovine transferrin [29], the aprotinin/transferrin complex could have been dissociated by treatment applied prior SDS-PAGE (heating in 2.5% SDS at 97 °C for 8 min). The subsequent observation of immunoreactivity in the low molecular weight (< 20.1 kDA) after Western blotting analysis of the 69 kDa fraction confirmed this hypothesis. Lastly, the formation

of aprotinin oligomers (dimer to decamer) in low and high salt concentration solutions (NaCl, KSCN and $(NH_4)_2SO_4$) has been described [30–32]. Although these associations appear to be improbable under physiological conditions [32], they could have occurred during the ammonium sulphate precipitation of intestinal extracts.

The low Ag1 yield obtained after purification including dialysis, desalting on Bio-Gel P-6DG and concentration steps could be explained by the unexpected low molecular weight of Ag1 in the form of free aprotinin.

As a consequence of the high basicity of aprotinin (pI 10.5), unspecific binding to negatively charged surfaces has been observed [16]. This could explain the low recovery (65%) of Ag1 immunoreactivity after gel permeation on Sephadex G100 column. Since heparin is a mixture of heterogeneous negatively charged polysaccharides, its binding to aprotinin resulted in acidic complexes characterised by a large negative charge heterogeneity (Fig. 4a, Fig. 8c). In immunoelectrophoresis and line-immunoelectrophoresis, differences in Ag1 mobility in crude heparin and intestinal or pulmonary extracts (Fig. 4) could be explained by: (i) a lower proportion of aprotinin complexed to heparin in tissue extracts than in crude heparin and (ii) the formation of ternary complexes, as observed in bovine mast cells between aprotinin, tryptase and heparin [33]. During anion-exchange chromatography of intestinal extracts, immunoreactive fractions were eluted at 0.02–0.12 and 1.20 M NaCl. As Ag1 was eluted at 1.20 M NaCl in crude heparin, it can be concluded that aprotinin/heparin complexes are present in tissue extracts. Different authors have observed unbound fractions (upto 15% of the total trypsin inhibitory activity of tissular extracts) during aprotinin purification on Mono S cation-exchangers [19,23]. These fractions are more likely aprotinin/heparin complexes than high molecular weight trypsin inhibitor, as hypothesised by these authors. In our studies, unbound fraction eluted from the Mono Q column was most probably free aprotinin.

Aprotinin belongs to the family of Kunitz-type serine protease inhibitors whose major target enzymes are trypsin, chymotrypsin, plasmin and kallikrein [21]. This easily explains the difficulties encountered for the tryptic digestion of the purified Ag1 fraction, and the particularly low peptide yield obtained.

In addition to BPTI which is by far the prevailing form in bovids, three glycosylated Kunitz-type inhibitors closely related to BPTI (BPTI-like inhibitor I, II and III; MW 7–10.6 kDa), have been identified in a number of bovine [19,22,23,34–36] and ovine tissues [37,38]. More recently, another BPTI-like inhibitor produced by chymotrypsin cleavage of a 56 kDa ovine Kunitz-type inhibitors has been localised in ovine connective tissues [20,39]. However, BPTI and BPTI-like inhibitors have been found only in small amounts in the ovine-species [37,38]. This could explain that only very small cross-reactivity has been detected with ovine and caprine crude heparins in the sensitive ELISA test developed for Ag1 quantitation in crude heparins [Levieux et al., J. Immunoassay, submitted]. Thus, Ag1 quantification in crude heparins using this immunoassay is essentially specific of the bovine species. Furthermore, the demonstration of Ag1 as an aprotinin/heparin complex is of special interest for the implementation of this ELISA. As aprotinin is localised in the mast cells together with heparin, any bovine tissues used for heparin purification can be detected. Moreover, aprotinin is highly resistant to heat, pH, chemical and enzymatic treatments, and consequently the processes used for crude heparin extraction are unlikely to destroy the antigenicity of Ag1 [16,40].

In conclusion, Ag1 is reinforced as a suitable target for an immunoassay dedicated to the control of crude heparins with regard to bovine contamination.

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