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Differentiation of bovine from porcine gelatines using polyclonal anti-peptide antibodies in indirect and competitive indirect ELISA

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Abstract

Gelatine is a collagen derivative obtained from bones and hides/skin mainly from bovine and pigs. As a consequence of the outbreak of bovine spongiform encephalopathy (BSE), the use of bovine gelatine in feed, food and pharmaceutical products has been restricted by regulatory authorities. However, no method was presently available for its specific detection. The large similarity in amino-acid sequences of collagens from different species make their immunochemical differentiation difficult when using polyclonal antibodies raised against the whole molecule [A. Venien, D. Levieux, J. Immunoassay Immunochem., in press]. To obtain bovine-specific antibodies, we immunized rabbits against putative species-specific sequences of the bovine collagen alpha 1(I) chain. Using these antibodies, an indirect ELISA was developed to allow a quick and easy differentiation between bovine and porcine gelatines. Moreover, a competitive indirect ELISA was found suitable to detect bovine gelatine in porcine gelatine purchased from laboratory chemicals suppliers down to a dilution of 2–4 parts per 1000 with CVs ranging from 5.7 to 7.7%. When testing mixtures of the largest possible range of industrial batches of bovine and porcine gelatines (skin/hides or bones origin, acid or alkaline processes, high or low Bloom) the detection limit was down to a dilution of 8 parts per 100 bovine gelatine in porcine gelatine. These ELISAs could be routinely used by pharmaceutical and food manufacturers to secure their supply chain.

Keywords: Gelatine; Bovine; Porcine; Bovine spongiform encephalopathy; BSE; ELISA; Peptides; Collagen

1. Introduction

Gelatine is a primary product used for quality improvement of foodstuffs and medicaments. In the food industry, gelatine can be found in innumerable products such as jelly, jellied meat, aspic, milk products like yoghurt, ice cream, desserts and sweets like gummy bears or marshmallows. The pharmaceutical industries use gelatine in soft and hard capsules, for binding in tablets, in form of sponges for treating wounds and as a colloid to expand the plasma after severe losses of blood.

Gelatine is a mixture of polypeptides obtained by partial hydrolysis of the collagen contained in bones and hides mainly from bovine and/or skins from pigs. However, the outbreak of BSE in the United Kingdom in 1986 and its extension to other countries have led the regulatory authorities to make restrictions to the use of bovine gelatine for human consumption, cosmetic and pharmaceutical products. In other respects, some religions ban the use of porcine gelatine for human consumption. Therefore, it became necessary to develop analytical methods intended to control the species origin of gelatine.

Due to the large similarity in structure and properties of gelatines from different origins, physicochemical methods based on principal component analysis of amino-acid residues obtained after acid hydrolysis [1] or calcium phosphate precipitation test [2] have not been proved able to detect mixtures of bovine gelatine in porcine gelatine.

Immunochemical methods have been proved very useful for the control of the species origin of animal tissues in food [3] and pharmaceutical products [4–7]. Hofmann et al. [8] examined an ELISA kit for its applicability to species identification in gelatine and gelatine-containing products, such

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as gum confectionery and processed turkey and chicken. Results were influenced by gelatine type, gelatine quality and concentration used and, in some cases, led to false negative or positive readings. Thus, the authors concluded that this ELISA cannot be reliably used for species identification of commercial gelatines.

Due to the very high homology between collagen sequences of mammals, raising species-specific antibodies is a major challenge. Moreover, the immunogenicity of gelatine is very low and the molecule should be chemically modified for raising antibodies in rabbits [9]. We have produced high titre antibodies against tyrosylated bovine and porcine gelatines (Venien and Levieux, J. Immunoassay Immunochem., submitted for publication). However, such antibodies were found highly sensitive to the alkaline or acidic process used for the gelatine production and not enough species-specific to allow a sensitive detection of mixture of low concentration of bovine gelatine in porcine gelatine.

We report here on the successful production of bovine-specific antibodies by immunization of rabbits with synthetic peptides mimicking a short putative species-specific sequence of the bovine alpha 1(I) chain. This allowed us to develop two ELISAs suitable for the differentiation of bovine from porcine gelatines and for the sensitive quantitation of their mixtures.

2. Materials and methods

2.1. Chemicals

Freund's complete and incomplete adjuvants, human serum albumin (HSA), *N*-acetyl-L-tyrosine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC), *N*-hydroxysuccinimide (NHS), 2-mercaptoethanol, hydroxylamine, collagen type I from calf skin, gelatine type B from bovine skin (75 Bloom) and gelatine type A from porcine skin (300 Bloom) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Gelatine for microbiology (porcine skin origin) was obtained from Merck Eurolab (45250 Briar-le-Canal), gelatine powder and TPMax gelatine (bovine origin) from Prolabo (VWR International, 94126 Fontenay sous Bois, France).

Samples of industrial batches of bovine and porcine gelatines were kindly donated by Rousselot (84808, L'Isle-sur-la-Sorgue, France). Their characteristics are described in Table 1.

Peroxidase-labelled goat IgG against rabbit IgG was purchased from Nordic (Tebu, 78510 Le Perray en Yvelines, France) and Sure Blue TMB from KPL (Thermolab Systems, BP249, 95615 Cergy-Pontoise, France).

2.2. Synthetic peptides

Two putative bovine-specific sequences of the bovine collagen were synthesised by solid-phase chemistry. Peptide 1 (Glu-Phe-Asp-Ala-Lys-Gly-Gly-Gly-Pro-Gly) was the N-terminal sequence of the telopeptide of the alpha 2(I) chain. Peptide 2 (Gly-Pro-Ala-Gly-Ala-Pro-Gly-Pro-Pro-Gly) was a sequence of the alpha helix of the alpha 1(I) chain. For antibody production, each peptide was cross-linked to hemocyanin from *Megathura crenulata* (keyhole-limpet hemocyanin, KLH) using glutaraldehyde as a coupling agent [10].

2.3. Tyrosylation of bovine and porcine gelatine

Tyrosine was activated by adding 30 mM EDAC and 60 mM NHS to a 300 mM solution of acetylated tyrosine in 10 mM phosphate buffer pH 6.0. The mixture was incubated 15 min at room temperature and EDAC was then quenched by adding 0.05 ml 2-mercaptoethanol. The activated tyrosine was diluted 3 mM in 10 mM phosphate buffer pH 6.0.

Table 1
Characteristics of the industrial batches of gelatines and their reactivity in indirect ELISA when tested with rabbit anti-peptide 2 (second boost)

Gelatine no.	Species	Tissue	Process	Viscosity (mPa s)	p*I*	Bloom		Indirect ELISA OD 450 nm
1	Bovine	Bone	Acid	3.20	7.3	High	231	4.808
2	Bovine	Bone	Acid	1.50	6.6	Low	50	4.579
3	Bovine	Bone	Lime	4.54	4.9	High	274	4.928
4	Bovine	Bone	Lime	2.10	4.9	Low	93	4.599
5	Bovine	Hide	Sodic	4.87	5.0	High	206	5.622
6	Bovine	Hide	Sodic	1.60	4.9	Low	40	4.642
7	Bovine	Hide	Acid	3.30	6.7	High	289	4.535
8	Bovine	Hide	Acid	1.55	5.4	Low	46	4.579
9	Bovine	Hide	Lime	4.64	5.1	High	228	4.505
10	Bovine	Hide	Lime	1.83	5.0	Low	60	4.802
11	Porcine	Bone	Acid	2.95	7.4	High	240	0.598
12	Porcine	Bone	Acid	1.90	6.8	Low	110	0.448
13	Porcine	Bone	Lime	2.65	4.9	Low	86	0.304
14	Porcine	Skin	Acid	4.40	9.0	High	265	0.596
15	Porcine	Skin	Acid	2.00	8.0	Low	75	0.585

Microtitre plates were coated with bovine (nos. 1–10) or porcine (nos. 11–15) gelatines from bone or hide/skin origin and produced by lime or acid process. A peroxidase-labelled goat anti-rabbit IgG was used as a tracer antibody. Results are means of duplicates.

The industrial batches of bovine or porcine gelatines were mixed in equal parts. Solutions (2.7 ml) were prepared at 10 mg/ml in phosphate buffer 20 mM pH 7.5 and tyrosylated by adding 0.3 ml of the activated tyrosine. Incubation was done for 2 h at room temperature.

Coupling was stopped by adding 10 mM final hydroxylamine and excess of activated tyrosine was eliminated by chromatography on a PD10 column (Amersham Biosciences, 91898 Orsay, France). The coupling efficiency was monitored at 280 nm.

2.4. Polyclonal antibodies

Bovine and porcine tyrosylated gelatines and synthetic peptides cross-linked to KLH were diluted to 1 mg/ml in sodium phosphate buffer (pH 7.2; 0.01 M) containing 0.15 M NaCl (PBS). Rabbits (four for each antigen) were immunized three folds at monthly intervals by multiple intradermal injections of antigen–adjuvant mixture prepared by emulsifying 1 ml antigen sample with 1 ml complete (first injection) or incomplete (booster injections) Freund's adjuvant [11]. Animals were bled 7–9 days after each booster injection and the sera were analysed for antibody activity and specificity by indirect and competitive indirect ELISA.

2.5. Indirect ELISA

Polystyrene flat-bottomed microtitre plates (Maxisorp, Nunc, Denmark) were coated overnight at room temperature or 2 h at 37 °C with 100 μl of bovine or porcine gelatine in PBS. The optimum coating concentration and pH were determined by checkerboard titration. All subsequent steps were performed at room temperature. Plates were washed five times with PBS containing 0.1% (v/v) Tween 20 (PBSTw). When blocking, the plates were incubated for 30 min with 200 μl of 0.5% HSA in PBS. After five washes with PBSTw, 100 μl of adequately diluted rabbit anti-peptide antiserum was added to the coated wells. After mixing, the plates were incubated for 1 h and washed five times with PBSTw. One hundred microlitres of adequately diluted peroxidase-labelled goat anti-rabbit IgG were added and incubated for 1 h in the dark. After five washes with PBSTw, 100 μl of TMB substrate was added, running the colour reaction for 30 min before stopping it with 0.36 M H_2SO_4 (50 μl/well). Absorbance was measured at 450 nm using a microplate reader (IEMS, Labsystem, Finland). PBSTw with 0.1% (v/v) HSA (PBSTw-HSA) was used as a diluent for the solutions. All analyses were made in duplicate.

2.6. Competitive indirect ELISA

Microtitre plates were coated, blocked and washed five times with PBSTw as described for indirect ELISA. Similarly, PBSTw-HSA was used as a diluent for the gelatines and antibodies solutions. While coating and blocking, one volume of the adequately diluted antiserum was incubated in 1 ml test tubes with one volume of serially diluted solutions of gelatines. After 60 min incubation, 100 μl of the antigen–antibody mixture were added to the wells for a 1 h incubation. After five washes with PBSTw, 100 μl of adequately diluted peroxidase-labelled goat anti-rabbit antibody was added to the wells and the plates were incubated for 1 h in the dark. All subsequent steps were unchanged. All analyses were made in duplicate and the detection limit was calculated as the mean blank signal minus 3S.D.

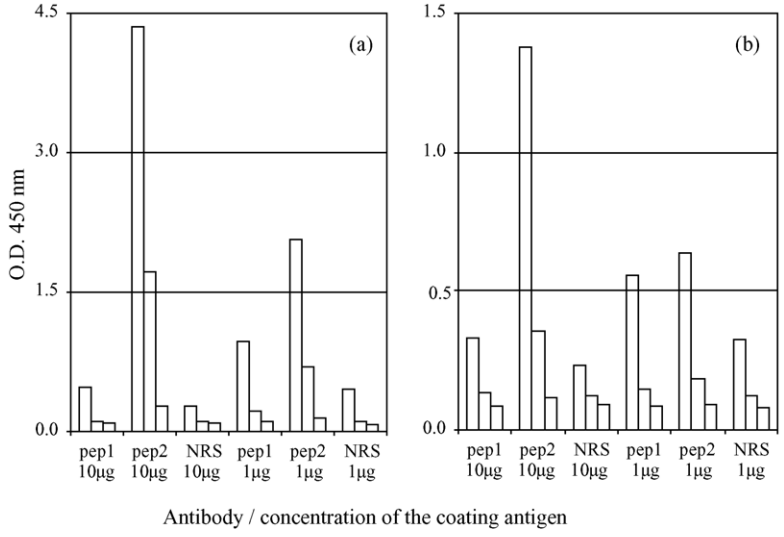

Fig. 1. Quantitation by indirect ELISA of the antibody titre of rabbits anti-peptides against collagen (a) and gelatine (b). Microtitre plates were coated with collagen I or bovine gelatine Sigma at 10 or 1 μg/ml. The anti-peptide 1 (pep1) and anti-peptide 2 (pep2) antisera obtained, respectively, at the second boost and first boost were tested diluted 10^{-3} (first columns), 10^{-4} (second columns) and 10^{-5} (third columns). A peroxidase-labelled goat anti-rabbit IgG was used as a tracer antibody. A normal rabbit serum (NRS) was used as a negative control. Results are means of duplicates.

3. Results

3.1. Reactivity of the polyclonal antibodies raised against synthetic peptides

A large variability of antibody response was observed among rabbits by indirect ELISA using the synthetic peptides coated at 0.1 μg/ml (not shown). Optical densities ranged from 0.3 to 3.0 and from 0.8 to 3.7 at a 10^{-5} dilution of the antisera when tested against peptide 1 and peptide 2, respectively. Maximal antibody titres were obtained at the first or second boost.

Reactivity against bovine collagen was essentially obtained with the antisera raised against peptide 2 (Fig. 1a). Optimal coating conditions were a 10 μg/ml concentration at pH 7.2 or 9.2.

Bovine gelatine from Sigma was also recognized quite exclusively by the antisera raised against peptide 2 (Fig. 1b). Optimal coating concentration was ≥10 μg/ml.

3.2. Characterization of the species origin of the gelatines using indirect ELISA

When testing bovine and porcine gelatines available from laboratory chemicals suppliers, the different batches were optimally coated at 50 μg/ml in PBS pH 7.2. High optical densities were observed with an anti-peptide 2 antiserum obtained at the first boost when tested against the two batches of bovine gelatine while no significant reactions were obtained against the four batches of porcine gelatine (Fig. 2a). When using a rabbit antiserum against bovine tyrosylated gelatines, no major difference in reactivity was obtained between the batches of bovine and porcine gelatine (Fig. 2b). Thus, the difference in reactivity observed with anti-peptide 2 between bovine and porcine gelatines could not be attributed to species difference in their coating capacity.

With the industrial batches of bovine and porcine gelatine coated at 50 μg/ml in PBS pH 7.2, similar results were obtained with anti-peptide 2 antiserum, since optical densities were ≥4.0 for bovine gelatines and ≤0.5 with porcine gelatines (Table 1).

Thus, indirect ELISA performed with anti-peptide 2 antiserum allowed a clearcut differentiation between all the bovine and porcine gelatines when used as coating antigens. However, when dilutions of bovine in porcine gelatines were made before their coating in the microtitre plate, the results were partially disappointing. The detection limit ranged between 15 and 35% or between 3 and 15% when the bovine gelatines were diluted in porcine acid bone or porcine limed bone gelatine, respectively. With the porcine acid skin gelatine used as a diluent, the detection limit was around 50% (results not shown).

3.3. Competitive indirect ELISA for the quantitation of bovine gelatines in porcine gelatines

Optimal concentrations of the bovine gelatine from Sigma used for the coating, of the rabbit antibody against peptide 2 and of the anti-rabbit conjugate were defined by checkerboard titration. Microtitre plates were optimally coated with bovine gelatine at 50 μg/ml in PBS buffer, the primary antibody and the conjugate were used diluted 1:5000 and 1:2000, respectively. Bovine gelatines were diluted in porcine gelatines at 5 mg/ml. In these conditions, the detection limit (mean blank signal minus 3S.D.) was established at 4 parts per 1000 bovine gelatine Prolabo in porcine gelatine Prolabo or 2.3 parts per 1000 (w/w) gelatine Sigma in porcine gelatine Sigma (Fig. 3). The intra-assays CVs obtained for 20 determinations of the Sigma and Prolabo bovine gelatines at 100 μg/ml in porcine gelatine at 5 mg/ml were found at 5.7 and 12.7% of the concentration, respectively.

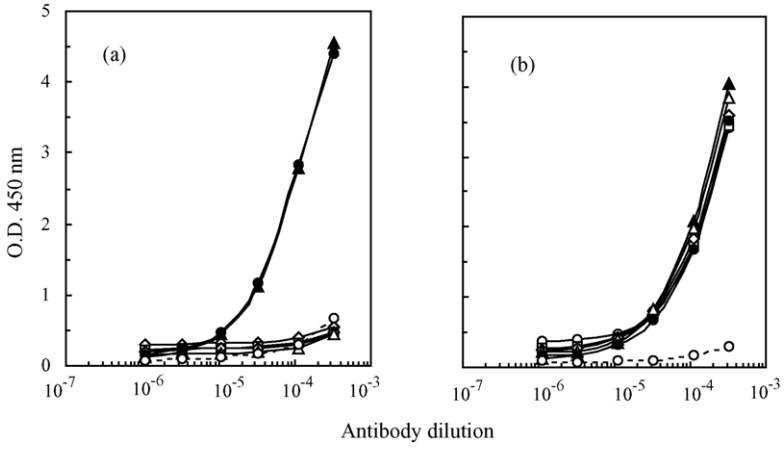

Fig. 2. Quantitation by indirect ELISA of the antibody titre of rabbit anti-peptide 2 (a) and rabbit anti-tyrosylated bovine gelatine (b) against gelatines purchased from laboratory chemicals suppliers. Microtitre plates were coated with bovine (closed symbols) or porcine (open symbols) gelatines from Sigma (circles), Prolabo (triangles), Merck (squares) or TPMax (lozenges) at 50 μg/ml; no coating (control): open circle with dashed line. A peroxidase-labelled goat anti-rabbit IgG was used as a tracer antibody. Results are means of duplicates.

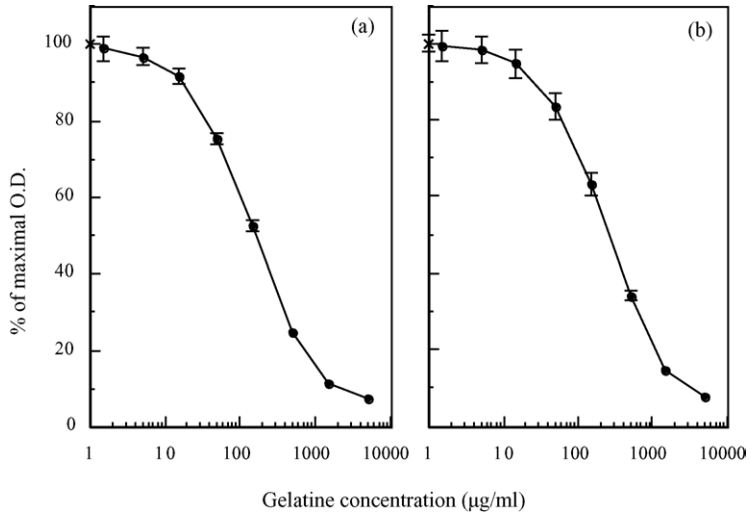

Fig. 3. Quantitation by competitive indirect ELISA of bovine gelatines in porcine gelatines. Microtitre plates were coated with bovine gelatine Sigma (a) or bovine gelatine Prolabo (b) at 50 μg/ml. Rabbit anti-peptide 2 obtained at the first boost was used as a primary antibody and peroxidase-labelled goat anti-rabbit IgG as a tracer antibody. Competitive antigens were bovine gelatine Sigma (a) or Prolabo (b); (×) no competitive antigen. Results are means ± S.D. ($n = 20$).

When testing industrial batches of bovine and porcine gelatines, a more or less marked inhibitory activity was observed with the porcine gelatines analysed at concentrations greater than 300 μg/ml (Fig. 4).

The detection limit of mixtures of high Bloom bovine and porcine gelatines obtained from the same tissue (skin/hide or bone) using the same kind of process (acid or alkaline) was down to 1.5–2 parts per 100 (w/w) bovine gelatine in porcine gelatine (not shown) when analysed at 300 μg/ml. Industrial batches of gelatines were successfully quantified by the competitive indirect ELISA (Fig. 4). However, some batches of porcine gelatines displayed an inhibitory activity when analysed at concentrations greater than 300 μg/ml (Fig. 5).

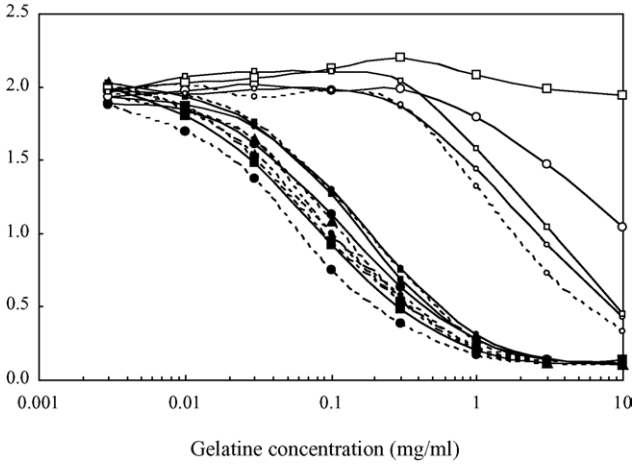

Fig. 4. Quantitation by competitive indirect ELISA of bovine and porcine industrial batches of gelatine. Microtitre plates were coated with bovine gelatine Sigma at 50 μg/ml. Rabbit anti-peptide 2 (first boost) was used as a primary antibody and peroxidase-labelled goat anti-rabbit IgG as a tracer antibody. Competitive antigens were bovine (closed symbols) or porcine (open symbols) gelatines obtained by acid (plain lines), lime (dashed lines) or sodic (triangles with plain lines) treatment of bones (circles) or hide/skin (squares and triangles). High Bloom: large symbols; low Bloom: small symbols. Results are means of duplicates.

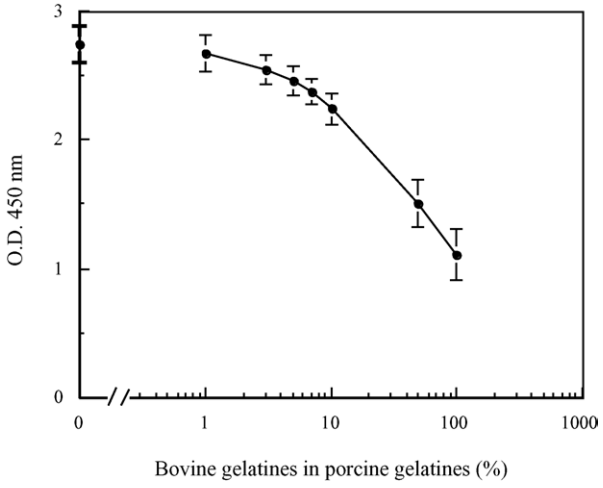

Fig. 5. Quantitation by competitive indirect ELISA of mixtures of bovine and porcine industrial batches of gelatine. Microtitre plates were coated with bovine gelatine Sigma at 50 μg/ml. Rabbit anti-peptide 2 was used as a primary antibody and peroxidase-labelleed goat anti-rabbit IgG as a tracer antibody. Competitive antigens were different percentages of bovine gelatines in porcine gelatines (1 in 11, 2 in 12, 4 in 13, 7 in 14 and 8 in 15) tested at 300 μg/ml. Numbers refer to full description of gelatines in Table 1. Results are means of the five inhibitions curves each obtained as mean of duplicate analyses.

4. Discussion

Over the last few years both national and international authorities have introduced legislation and regulations to enhance the safety of gelatine with regards to the transmissible spongiform encephalopathies (TSE) risks. In most

cases, these regulations concern a limitation of the country of origin for raw material, parts of the animals that can be used as raw material and value ranges for the physical processing parameters of gelatine. According to the Scientific Steering Committee of the European Union, the currently commonly used gelatine production processes have a considerable TSE infectivity reduction capacity exceeding 4.5 logs [12]. However, the experiments currently available to assess the TSE infectivity reduction capacity of a production process do not demonstrate a complete destruction of all TSE infectivity in a test sample. They determine a quantitative or semi-quantitative reduction in the amount of infectivity, the "clearance factor", which is limited by the sensitivity of the assay system and the starting titre of the TSE spiked material. It is thus not possible in the current state of knowledge to conclude that any given process for the production of ruminant-derived products would result in an end product that is completely free of TSE agent [13].

A method has been proposed to detect possible prion contaminants using Western blotting of PrP^{Sc} [14]. The method includes a collagenase digestion, a concentration step using PEG, the usual proteinase K digestion and a precipitation step with PEG. However, the system can detect no lower than 4×10^3–10^4 LD_{50} of mouse prion and is less sensitive than the time-consuming mouse bioassay with intracerebral injection.

To reduce the TSE risk, food and pharmaceutical companies have been progressively switching to porcine-sourced gelatine and the characterization of the animal origin of raw materials used for production of gelatine is now a major goal for manufacturers. However, to our knowledge no validated method for gelatine to determine the origin of the raw material is available yet.

We have recently obtained high titre antibodies upon immunization of rabbits with tyrosylated bovine and porcine gelatines (Venien and Levieux, J. Immunoassay Immunochem., submitted for publication). We have observed large differences in titre and species-specificity during the course of the immunization and among rabbits. Moreover, the antisera were highly sensitive to the alkaline or acidic process used for gelatine production. A competitive indirect ELISA has allowed to detect porcine acidic gelatines with around 10-fold higher sensitivity than their bovine counterparts. However, the porcine limed bone has not been detected by this ELISA.

These results prompted us to elaborate a strategy based on the production of antibodies against more defined species-specific amino-acid sequences. Major antigenic determinants have been localized in N-terminal and C-terminal non-helical sequences for which no function in formation or maintenance of the triple-helical structure is assumed [15]. Yet, conformation independent antigenic determinants have also been characterized in the central regions of the unfolded polypeptide chain having a helical conformation in the native molecule [15,16]. However, there is an exceptionally high homology between published sequences of collagen of different species. As an example, we calculated a 97% homology between the alpha 1(I) chain of bovine and human collagen. Moreover, the porcine collagen sequence is not presently available. Thus, we chose as putative bovine-specific sequences those which display amino-acid changes between bovine and unrelated species such as horse, human and dog. We finally selected one putative species-specific sequence from the N-terminal sequence (peptide 1) and another one (peptide 2) from the central region of the bovine alpha 1(I) chain.

Reactivity of the anti-peptide 1 antibodies was low when tested against collagen and gelatine. This would indicate a loss of the telopeptide during collagen extraction and gelatine production or differences in the three-dimensional conformation between the free synthetic peptide and the native N-terminal peptide of the collagen/e chain.

In contrast, the anti-peptide 2 antibodies reacted strongly in indirect ELISA with the panel of bovine gelatines coated in the microtitre plates while the porcine gelatines were not detected. This indirect ELISA could be used as a simple and quick identification test. However, this test cannot be used for the detection of low amounts of bovine gelatines in porcine gelatines since the results obtained when coating compatible mixtures of bovine and porcine gelatines were relatively disappointing. This phenomenon cannot be easily explained and could be related to changes in the three-dimensional structure of the mixed molecules during coating.

Such a phenomenon disappeared when analysing the same mixtures of gelatines by the indirect competitive ELISA. High sensitivities (2–4 parts per 1000) were obtained for the detection of bovine gelatines in porcine gelatines purchased from laboratory chemicals suppliers. The sensivity was 8 parts per 100 bovine gelatines in porcine gelatines when testing the largest possible range of industrial batches of gelatines covering both bovine and porcine skin/hide or bone origin, with acid or alkaline process and high or low Bloom. While all the bovine gelatines reacted quite similarly in the indirect competitive ELISA, the residual reactivity of the different batches of porcine gelatines was variable and seems apparently correlated with the denaturation effect of the process used for their production: acid skin high Bloom (no inhibition) < acid bone high Bloom < acid skin low Bloom < acid bone low Bloom < limed bone low Bloom. Thus, detection of bovine high Bloom gelatines in porcine high Bloom gelatines was more sensitive, down to 1.5–2%. However, the possibility of slight accidental contaminations of some batches of porcine gelatines by bovine gelatines in industrial sites which producing gelatines from the two origins cannot be totally excluded.

These detection limits allow an efficient control of the gelatine batches manufactured and the described ELISA could be routinely used by pharmaceutical and food manufacturers to secure their supply chain.

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