Immunological detection of osteocalcin in meat and bone meal: a novel heat stable marker for the investigation of illegal feed adulteration

Grit Kreuz, Jutta Zagon, Hermann Broll, Christian Bernhardt, Bettina Linke, Alfonso Lampen

To cite this version:

HAL Id: hal-00783932
https://hal.science/hal-00783932
Submitted on 2 Feb 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
## Immunological detection of osteocalcin in meat and bone meal: a novel heat stable marker for the investigation of illegal feed adulteration

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>Food Additives and Contaminants</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>TFAC-2011-420.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Research Paper</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>22-Nov-2011</td>
</tr>
<tr>
<td>Methods/Techniques:</td>
<td>Immunoassays, Traceability, Authenticity</td>
</tr>
<tr>
<td>Additives/Contaminants:</td>
<td>Animal products, Feeding</td>
</tr>
<tr>
<td>Food Types:</td>
<td>Animal products – meat, Animal feedingstuffs</td>
</tr>
<tr>
<td>Abstract:</td>
<td>A sandwich ELISA was developed for the detection of bovine meat and bone meal (BMBM) in feed, based on polyclonal rabbit antibodies raised against the synthetic N-terminal amino acid sequence 1–9 (YLDHWLGAP) of bovine osteocalcin. To set up a sandwich ELISA pair, a commercial mouse monoclonal capture antibody binding to a highly conserved epitope in the mid-fragment of the peptide was employed. It is shown that the bone marker osteocalcin is immunologically well detectable in BMBM extracts obtained by a simple EDTA based procedure even in a sample heated up to 145 °C. Furthermore a genus-specific restriction of the major specificity to cattle and horse was possible. The observed bi-specificity is consistent with theoretical predictions. The assay sensitivity with bovine osteocalcin of 1 ng was sufficient to enable the detection of 0.1% BMBM in compound plant feed or fish meal, for which no cross reaction was observed. In general the quantification of osteocalcin in extracts is possible using a standard curve procedure with pure bovine osteocalcin.</td>
</tr>
</tbody>
</table>
Immunological detection of osteocalcin in meat and bone meal: a novel heat stable marker for the investigation of illegal feed adulteration

G. Kreuz, J. Zagon*, H. Broll, C. Bernhardt, B. Linke, A. Lampen

Federal Institute for Risk Assessment, D-14195 Berlin, Germany

* Corresponding author. E-mail: jutta.zagon@bfr.bund.de

Abstract

A sandwich ELISA was developed for the detection of bovine meat and bone meal (BMBM) in feed, based on polyclonal rabbit antibodies raised against the synthetic N-terminal amino acid sequence 1–9 (YLDHWLGAP) of bovine osteocalcin. To set up a sandwich ELISA pair, a commercial mouse monoclonal capture antibody binding to a highly conserved epitope in the mid-fragment of the peptide was employed. It is shown that the bone marker osteocalcin is immunologically well detectable in BMBM extracts obtained by a simple EDTA based procedure even in a sample heated up to 145 °C. Furthermore a genus-specific restriction of the major specificity to cattle and horse was possible. The observed bi-specificity is consistent with theoretical predictions. The assay sensitivity with bovine osteocalcin of 1 ng was sufficient to enable the detection of 0.1% BMBM in compound plant feed or fish meal, for which no cross reaction was observed. In general the quantification of osteocalcin in extracts is possible using a standard curve procedure with pure bovine osteocalcin.
KEYWORDS: Osteocalcin, Meat-and-Bone-Meal (MBM), Bovine spongiform encephalopathy (BSE), Animal feed, Processed animal proteins (PAPs), ELISA, Species identification
Introduction

Feeding meat and bone meal (MBM) to farmed animals is prohibited in Europe by Regulation (EC) No 999/2001. In addition, an overarching species-to-species feed ban has been laid down by Regulation (EC) No 1069/2009. The only exemption from the strict MBM ban is fish meal, which has been allowed for pig and fowl feeding and recently also for calves in milk replacing feed mixtures (Regulation (EC) No 999/2001). According to the recitals of the above mentioned Regulation it might be assumed that the feed ban for ruminants will not be lifted in the near future whereas the prohibition of processed animal proteins (PAP) feeding to other animals than ruminants might be relaxed as far as analytical tools for species identification are available.

The official method for the detection of MBM in feeding stuff is based on classical light microscopy (Regulation (EC) No 152/2009). A second test may also be carried out using variant or alternative methods, in order to improve the detection of certain types of animal constituents or to further specify the origin of the animal constituents. The microscopic-based method reliably detects heat- and pressure-stable particles of animal origin like bone fragments, teeth, hair, gills or scales which are resisting the legally required MBM treatment of 133 °C for 20 min at 300 kPa (Liu et al. 2011). However, by applying the microscope approach it is not possible to distinguish between species or genus in samples containing PAPs. Thus, up to now the implemented species-to-species ban cannot be controlled.

Furthermore, the microscopic investigation depends solely on individual skills and allows an estimation but not quantification of the MBM content in a feed sample. Therefore - and against the background of recently discussed thresholds and a further lifting of the feed ban in the European Union (COMMISSION STAFF WORKING DOCUMENT 2010) - there is an urgent need for alternative methods which might overcome present drawbacks to enable legally consistent feed survey and law enforcement.
The immunological detection of PAPs is for long used as an alternative to microscopy. Immunoassays can be performed in any laboratory independently from sophisticated equipment or particularly trained staff. Antibody based assays are convertible into rapid formats like lateral flow assays (dip stick tests) representing most convenient field methods for rapid screening purposes. Ruminant or pork specific antibodies binding to PAPs, so far have been raised either directly against bovine meat and bone meal (MBM) (Muldoon et al. 2004; Kim et al. 2004), mixtures of muscle proteins (Kim et al. 2005; Chen et al. 1998; Ansfield 1994), purified troponin I (Chen et al. 2002) or h-caldesmon (Kim et al. 2004), isolated from skeletal or smooth muscle of the bovine intestine, respectively. Four commercial PAP detecting test kits (two dip stick tests and two ELISA) based on antibodies binding to the described targets have been repeatedly evaluated in studies conducted by official institutions in the USA or Europe. All of them revealed different shortcomings in terms of specificity, selectivity or sensitivity (Fumière et al. 2009). Moreover, from the viewpoint of validation, it is not satisfying that neither the kit manuals nor accompanying scientific publications disclose the precise nature of the targeted epitopes, neither amino acid sequences nor conformational molecular reaction sites, involved in the antigen-antibody binding. As a consequence, potential cross reactivity cannot be deduced in silico and the likelihood to reproduce immunization results obtained with changing or undefined antigen mixtures is low.

The polymerase chain reaction (PCR) is another alternative approach to identify animal species-specific components in feedstuffs. PCR is highly-specific and is amplifying target DNA sequences in an exponential way. However PCR is not tissue-specific due to the fact that the DNA of an individual organism does not differ between different tissues. Therefore it is not possible to distinguish if the detected DNA is derived from the use of MBM or from allowed animal products such as milk or egg protein.
In the present study we employed defined target sequences of bovine osteocalcin, consisting of the amino acid sequence 1-9 and the epitope 17 of the peptide, to establish an ELISA for the purpose of MBM detection in feed. Osteocalcin is a small extracellular bone matrix protein produced by osteoblasts and a common constituent in calcified bone tissue all over the vertebrate group including bone fish (Hauschka et al. 1989; Nishimoto et al. 2003; Laizé et al. 2005). The mature peptide of approx. 5800-5900 Da size is composed of 45 – 49 amino acids. Up to 90 % of osteocalcin is secreted inside the bone tissue where it is, apart from collagen, the most abundant bone protein accounting up to 2 mg/g bone matrix or 1 – 5 % (w/w) of the total bone protein, respectively 10 – 20 % (w/w) of the non-collagenous bone proteins (Hauschka et al. 1989). A minor share of osteocalcin (10 - 40 %) is circulating in the blood where it is - once detached from the bone mineral - quickly degraded in kidney and liver (Lee et al. 2000). The function of osteocalcin is not completely understood but the molecule seems to play a role in the molecular organisation of the hydroxyapatite skeleton as well as in bone turnover (Dowd et al. 2003; Ducy et al. 1996). Three glutamic acid residues are located at amino acid position 17, 21 and 24, constituting a highly conserved motif of the peptide. These glutamic acids are transformed into γ-carboxyglutamic acids in a post-translational carboxylation step and function as precipitation sites for Ca$^{2+}$ ions. In the presence of calcium the molecule forms a compact structure comprising of two α-helical regions and a hydrophobic core near the C-terminus (Dowd et al. 2003). Both, N- and C-terminal sequences of osteocalcin reveal species specific differences (Figure 1).

Osteocalcin is known to be heat stable. Human or bovine bone osteocalcin which was extracted at 120°C overnight, lyophilized and decarboxylated at 110 °C for five hours, still retained its substrate specificity towards enzymatic vitamin K-dependent carboxylase (Vermeer et al. 1984). Osteocalcin could be identified in 75.000-year-old Neanderthal bone by MALDI-TOF/TOF-MS (Nielsen-Marsh et al. 2005) and in fossil bones and teeth by immunoassay-based investigation (Ulrich et al. 1987; Collins et al. 2000; Muyzer et al. 1992).

We recently demonstrated that osteocalcin can be identified and differentiated in MBM of
bovine and pig origin using high-resolution (MALDI/TOF-MS and HR-ESI Q/TOF) mass spectrometry (Balizs et al. 2011).

The aim of the present study was to investigate i) if osteocalcin is still immunologically detectable in MBM treated according to the legal requirements, ii) how far a discrimination of farmed animals, especially cattle in feed, is possible and iii) to evaluate if a selectivity in an ELISA down to 0.1 % MBM either in compound feed or fish meal would be achievable.

Materials and Methods

Materials

Pure bovine osteocalcin (OC) was purchased from Merck Calbiochem (Merck, Darmstadt, Germany). Bone from farmed animals and pasteurized egg- and milk powder were purchased from local retailer in Berlin. 100 % pure bovine meat and bone meal (BMBM), treated 145 °C, with a bone content of approx. 80 %, as well as 100 % sheep meat and bone meal (OMBM) were kindly submitted by the European Reference Laboratory for Animal Proteins EU-RL AP, Gembloux, Belgium. From the same source a spiked 0.1 % BMBM sample in plant feed (Plant Feed-0.1 % BMBM), serving as reference and training material for microscopists, was obtained. This material was purity checked by classical microscopy and PCR. Based on the results the EU-RL AP specified the material as a mix of four pure bovine meat and bone meals from different heat treatments with a bone content of ~ 54 %.

On request, the EU-RL AP also kindly made available a sample of the non-contaminated plant feed background (Plant Feed-0 % BMBM), used for spiking consisting of a classical feed mix containing wheat, tapioca, soybean, rapeseed, palm kernel and barley meals, beet pulp, molasses, bakery by-products, vegetable fat, limestone, salts and vitamins. A commercial dog feed BMBM (100 % cattle according to the label) was purchased from PerNaturam V.O.F. (Aalten, The Netherlands). Fish meal (FM) and poultry meal (PM) produced under unknown processing parameters were obtained from diverse rendering plants in Germany (SARIA Bio-Industries GmbH & Co., Selm, Germany). An anonymous
sample of BMBM treated in accordance with legal requirements (133 °C) was obtained from the Department of Feed and Feed Additives of the Federal Institute for Risk Assessment, Berlin. Pure pig meal from an anonymous source was kindly supplied by LUFA Nord-West (Oldenburg, Germany). Samples containing 0.1, 1 and 10 % (v/v) BMBM (dog feed) extract were prepared by mixing respective volumes of MBM extracts containing a comparable total protein concentration.

Sample preparation and protein extraction

For the production of reference proteins, native bones from different species were cleaned from any fat, meat residues and bone marrow and roughly trenched. Bone fragments were degreased by Soxhlet extraction in a Soxtec™ 1045 apparatus (FOSS GmbH, Rellingen, Germany) with boiling petroleum ether (Merck, Darmstadt, Germany). The defatted material was transferred into steel cartridges, cooled down for 2-3 minutes with liquid nitrogen and ground to yield a fine powder using a steel ball mill (TissueLyser Qiagen, Cat.No.85220, Retsch GmbH, Haan, Germany) for 3 min and 30 Hz. MBM, plant feed or bone powder was extracted by EDTA-extraction. All centrifugation steps were performed at 15000 g, 10-20 min, 4 °C in a Heraeus Multifuge® 3S+/3SR+ (Thermo Scientific, Schwerte, Germany). In brief, 2 g sample were extracted with 8 ml 0.5 M Ammonium EDTA (pH 6.1) by stirring at 4 °C over night. The slurry was transferred into a conical Falcon™ tube and centrifuged. The supernatant was transferred into ultrafiltration tubes with an exclusion size of 3000 Da (Amicon® Ultra Centrifugal Filters Ultracel-3K, Millipore, Ireland) and the retained protein fraction was washed with 2 ml phosphate buffered saline (PBS, 10 mM NaCl, 10 mM KCl, 10 mM Na₂HPO₄,NaH₂PO₄, pH 7.4) to exchange the buffer.

The protein concentration was measured for all extracts in triplicates using a Bicinchinonic Acid Protein Assay Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer’s instruction. All extracts (Table 1) were aliquoted and stored at - 80 °C until use.
Antibodies

A commercial mouse monoclonal antibody binding to the epitope aa17 of bovine osteocalcin (OC4-30, Takara Bio Inc., Otsu/Shiga, Japan), specified to cross react with human, bovine, rabbit, sheep, dog, monkey and goat but not rat or mouse, was used as animal specific capture antibody in a two-side sandwich ELISA. Polyclonal antibodies (PAB) were raised in two rabbits against the synthetic and LPH- (Limulus polyphemus hemocyanin) coupled osteocalcin amino acid sequence OC 1-9 (YLDHWLGAP) by a service laboratory (Biogenes, Berlin). The best performing serum in a pre-test against BSA-coupled amino acid sequence (PAB OC1-9) was purified by affinity chromatography on CNBr-activated sepharose by the service laboratory to isolate a monospecific IgG-fraction which was used in the sandwich ELISA as secondary antibody. For this purpose PAB OC1-9 was conjugated with streptavidin (PAB OC1-9-Strep) using a commercial kit according to the manufacturer’s instruction (Lightning-Link™ Streptavidin Conjugation Kit, Innova Biosciences, Cambridge, UK, Technical Bulletin 1086, Release 02, June 2010).

Sandwich ELISA

Capture, secondary and detecting agent concentrations as well as the incubation time and temperature were optimized in grid experiments. The final protocol was performed as follows. Washing steps were repeated in general four times with 380 µl PBS-Tween 20 (154 mM NaCl, 7.7 mM Na₂HPO₄, 2 mM NaH₂PO₄, 0.05% Tween 20, pH 7.4) using an automatic ELISA Washer (Wellwash, Thermo Fisher Scientific). All incubation steps were carried out in a volume of 100 µl/well. 1 µg/ml of the capture antibody (OC4-30) was bound to a microtiter plate (Maxisorp, Nunc™, Denmark) for 48h at 4°C. The plate was washed and then blocked by adding 200 µl/well Blocking Buffer made of 1.5% (w/v) bovine albumine fraction V (BSA; Merck, Darmstadt, Germany) in 10 mM PBS, pH 7.4, overnight at 4°C. The plate was washed and incubated with standard dilutions in PBS (1, 10, 100 and 1000 ng/ml osteocalcin) or undiluted sample extract (Table 1) for 2 h at room temperature (RT). The
plate was washed and incubated with detection antibody (PAB OC1-9-Strep; 1:500 in PBS) for 2 h/RT, washed and incubated 1 h/RT with Biotinylated HRP (Invitrogen Corporation, Camarillo, CA; 1:10.000 in PBS). The plate was washed and the assay developed with chromogenic solution prepared as follows: 15 ml of citrate buffer (210 mM citric acid monohydrate, 300 mM KOH, pH 3.95) and 13.6 mM freshly added H$_2$O$_2$ were mixed 20:1 (v/v) with 21.6 mM TMB (3,3',5,5'-Tetramethylbenzidine, BioChemica, AppliChem, Darmstadt, Germany) diluted in 2.5 ml acetone and 22.5 ml methanol (p.A.). After incubation for 20 min/RT in the dark the reaction was stopped by adding 1N H$_2$SO$_4$ to each well. The optical density was determined at 450 nm against air in a multimode plate reader (Mithras LB 940, Berthold Tech. GMBH & Co. KG, Bad Wildbad, Germany). All samples and standards were measured in triplicates, statistical relevance between two data sets was calculated by two-side unpaired Student’s t-test.

**SDS-PAGE and Western blot**

Discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Tris-Glycin buffer according to Laemmli (1970) using a Mini Protean® Tetra System electrophoresis cell (Bio-Rad, Laboratories Inc., Germany). For the separation of low molecular weight proteins the separating gel contained 16.5% and the stacking gel 7.5% acrylamide (Rotiphorese® Gel 30 [37.5:1]), Carl Roth GmbH & Co KG, Karlsruhe, Germany). Separated proteins in the gel were electro-transferred onto a nitrocellulose membrane (Amersham™Hybond™–ECL, GE Healthcare) with a blotter unit (Bio-Rad, Laboratories Inc.) at 4°C and 350mA/400V for 1 h using transfer buffer as described by the blotter unit’s manual. The blot was blocked in PBS/3 % BSA for 45 min at RT followed by an overnight incubation at 4°C with PAB OC1-9-Strep diluted 1:500 in PBS/1.5 % BSA. The membrane was washed three times with TBST and PAB OC1-9-Strep binding onto the blot was detected by biotinylated HRP (1:5.000 in PBS containing 1.5 % BSA) after 1 h/RT incubation. After washing the blot was developed by adding 750 µl of a ready-mixed developing solution (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and chemo
luminescence was measured with the Imaging System VersaDoc MP 4000 (Bio-Rad Laboratories, Munich, Germany).

Results

In order to develop a sandwich ELISA for the detection of processed bovine osteocalcin (OC), a rabbit polyclonal antibody was raised against the amino acid sequence 1-9 of pure bovine osteocalcin. The best performing pre-tested serum (PAB OC1-9) was monospecifically purified. To confirm the reactivity of the isolated mono-specific IgG fraction, an immunoblot was carried out with an EDTA protein extract produced from native bovine bone.

Solely one major low-molecular weight protein band (~5.8 kDa) was detectable corresponding to the size of intact mature reference protein (Figure 2). No significant unspecific binding to other bone proteins occurred in bovine bone extract so that the antibody PAB OC1-9 could be used to establish a sandwich ELISA pair with a monoclonal mouse capture antibody (OC4-30).

The intra- and inter-assay variability and the sensitivity of the final ELISA set up was validated with standard dilutions of bovine osteocalcin in triplicates on three different days by the same operator (Figure 3). According to this validation experiment, the two-side assay is characterized by an average background signal ranging between $A_{450\text{nm}}$ 0.170 – 0.260 for the non template control (NTC; PBS instead of sample) and blank (PBS instead of capture MAB). A significant signal above NTC and blank was obtained - with a probability of $p < 0.05$ in two-side Student’s t-test - starting from an OC amount of 1 ng per well. Above this value the standard deviation is rising along with increasing signal strength, but the calculated inter assay coefficient of variation (2.8 – 23.3 %) (Figure 3) was considered satisfactory for all concentration levels and the assay accepted for the scope of investigation.

EDTA extracts of native bone derived from most significant farmed animals as well as egg and milk protein and a compound plant feed used for BMBM spiking (Plant Feed-0 % BMBM)
were measured to evaluate the specificity of the test. All EDTA-extracts were applied undiluted in ELISA, to ensure a maximum achievable response with highly processed materials or matrices of low MBM content. Protein yields typically varied between 0.6 – 2.8 mg/ml for different bone and MBM extracts (Table 1). The EDTA extract with the highest protein amount was gained from egg powder with approximately 4 mg/ml.

The results of the specificity testing are summarized in Figure 4. The Limit of detection (LOD) was calculated as the mean absorption $A_{450}$ value plus 3-fold standard deviation of 13 different negative samples (measured in triplicate) which constitute typical feed compounds (plant feed, whole milk powder, hen’s egg) or potential contamination with other species than cattle (fish meal, poultry meal, sheep MBM, sheep bone, pork MBM, pork bone, chicken bone, goat bone, rabbit bone) as well as the NTC. The horse sample has been excluded from the calculation due to the 100 % amino acid identity with bovine osteocalcin and could therefore not been considered as a negative sample. In all following experiments the LOD indicates the baseline to discriminate true positive from negative results. The strongest ELISA signals ($A_{450}$ nm > 3) were detected for cattle and horse bone extracts. For these samples absolute protein amounts of 55 to 75 µg/reaction were sufficient to elicit a strong ELISA response with signals rapidly out-of-range if using further increased input amounts.

Regarding bone extracts from other animals, a weaker but significant ELISA signal pointing to an evident cross reaction was measured with 100 % native pork bone extract. In a western blot investigation with pure pig bone extract and PAB OC1-9 it was noticed, that a cross reaction is directed against an unidentified protein of approximately 31 kDa (data not shown). In addition, a weak cross reaction was also monitored for 100 % native sheep bone extract (Figure 4). Bone extracts from chicken, goat or rabbit so as EDTA-extractable proteins from hen’s egg, whole milk powder or compound plant feed all yielded negative results below LOD-Baseline. EDTA-extracts derived from goose- or salmon bone, feather meal, as well as rye, maize and soybean were tested negative (data not shown).
In the next step, the capacity of the immunoassay to detect OC in heat treated meat and bone meal extracts was investigated, to test the applicability of the ELISA on real life samples. Since no horse MBM was available, horse serum was used instead as an exemplary matrix containing only trace amounts of osteocalcin. The total protein amount of all employed undiluted MBM-extracts per ELISA well ranged in a comparable order of magnitude between approx. 100 (poultry meal) – 280 µg (BMBM). The results are depicted in Figure 5. All BMBM samples showed a pronounced immunological response, even if the material was heated up to 145°C, whereas fish, poultry and sheep meal were tested negative. Therefore the cross reaction with native sheep bone is judged negligible since it is not interfering with the scope of the assay. A positive reaction was only detectable for undiluted horse serum and – in contrast to sheep meal - for pork MBM extract. Though the immune response against porcine MBM was rather weak compared with BMBM (c.f. dog feed), the obtained pork MBM signal exceeded the LOD-baseline (Figure 5).

The selectivity of the test was evaluated with extracts yielded from a standard plant feed spiked with 0.1 % (w/v) BMBM in comparison with the non-spiked matrix. In addition, since no homogeneity approved material was available, dilutions of 0.1 % BMBM (dog feed) in fish meal extracts were measured (Figure 6). The test discriminated 0.1 % (w/v; v/v) BMBM-spiked plant feed or fish meal, respectively, from the non-spiked matrix. The quantitative content of osteocalcin in the plant feed spiked with 0.1 % (w/v) BMBM corresponds to 6.7 ng calculated in relation to the standard curve. Due to the observed cross reaction of the ELISA with pig meal, a separate selectivity experiment was performed. 0.1 %, 1 % and 10 % (v/v) BMBM (dog feed) extract in pig meal extract was measured on three days (Table 2). A significant positive response was measurable for the 10 % (v/v) BMBM spiking level but not for the 1 % (v/v) spiked sample. The absorption measured with 100 % pork meal extract did not exceed the values corresponding to 10 ng bovine OC per well.

**Discussion**
A pre-requisite for the development of an immunological assay to detect fraudulent application to or unintended traces of MBM in feed is the thorough choice of a protein which exhibits exceptional resistance against mechanical forces, pressure and peak temperatures up to 140 – 150°C evoked by the rendering process (Pérez-Calvo et al. 2010; Garcia et al. 2006). In this study the heat stable bone protein osteocalcin has proved to be a well suited MBM marker since a strong immunological signal was still detectable in BMBM even if processed at 145°C. In addition, osteocalcin represents a genuine MBM- as well as animal marker protein since it is exclusively expressed in bone tissue or dentin in all vertebrates including bone fish (Hauschka et al. 1989).

Though fairly stable if bound to the hydroxyapatite skeleton, osteocalcin under natural conditions undergoes rapid degradation in a turnover process in the blood plasma. Two arginine-arginine cleavage sites are situated at the amino acid positions 19(Arg)-20(Arg) and 43(Arg)-44(Arg) (Figure 1) resulting in two to three fragments (aa1-19, aa20-43, aa44-49) on tryptic hydrolysis. The cleavage site near the C-terminal sequence is quickly hydrolysed cutting off the latter six amino acid residues whereas the resting aa1-43 fragment (N-MID fragment) is far more stable (Lee et al. 2000). Respecting the binding sites of the antibodies in our assay (aa1-9 and the epitope aa17) the results obtained with three different bovine MBM samples underline that a significant portion of at least the N-MID (aa1-43) is still immunologically accessible in MBM.

To restrict the assay specificity to cattle, the synthetic N-terminal amino acid sequence aa1-9 of bovine osteocalcin was used for the immunization of rabbits to exclude other most relevant farmed animals. The N-terminal sequences of osteocalcin in the genus Bos, Sus, Ovis and Capra differ only in one to three amino acids whereas this sequence is completely identical among Bos and Equus (Figure 1). The specificity testing confirmed that minor sequence differences were sufficient to limit the major polyclonal antibody reactivity to Bos with the predictable exception of Equus. However, an increasing tendency towards cross reaction is
observed in accordance with the number of amino acid exchanges in relation to *Bos* OC aa1-9 ranking from *Capra* (3 exchanges): no cross reaction – *Ovies* (2 exchanges): very weak cross reaction – *Sus* (1 exchange): weak cross reaction - *Equus* (100 % sequence identity): strong cross reaction. The observed binding of the polyclonal rabbit IgG fraction to a 31 kDa-protein in western blot studies with PAB OC1-9 and porcine bone extract (data not shown) points rather to an unspecific side reaction to another protein than osteocalcin. To unravel the nature of this cross reaction a mass spectroscopic investigation would be required which was not subject of this study. However, the exchange of the voluminous aromatic amino acid tryptophan at position aa5 in bovine OC against glycine in *Sus* could be targeted by the production of a high affinity monoclonal antibody.

In the case of horse and cattle a sequence difference between both is given only at position aa19 where lysine in bovine osteocalcin is exchanged by arginine in the genus *Equus* (Figure 1). Therefore elimination of cross reaction in theory could be possible by an exchange of the capture antibody. However, an attempt to boost a polyclonal rabbit capture antibody against the synthetic mid-fragment spanning aa16 – 22 of bovine osteocalcin was not successful. This might be due to the lacking carboxylation of the glutamic acid residues at position 17, 21 and 24 of the synthetic peptide used for immunization, preventing appropriate molecule folding. In addition the hydrophobic core of the molecule (Dowd et al. 2003) most probably requires a conformational epitope rather than a sequence based recognition site for antibody binding. The latter consideration is supported by a study using monoclonal and polyclonal OC- directed antibodies which were tested with differently sized fragments of the molecule. The authors demonstrated a generally poor affinity of antibodies against OC fragments comprising solely of aa11-29 or aa21-43 (Gundberg et al. 1998). Accordingly, the exclusion of horse- but also a reduction of pig cross reactivity could be tackled by a compelling anti-protein production mode, e.g. as given by stringent phage display or monoclonal selection against native bovine osteocalcin fragments aa1-43, aa1-19, aa 4-19 or intact osteocalcin. The latter is planned for further investigations.
Apart from osteocalcin, $\gamma$-carboxylated glutamic acids are occurring e.g. in proteins involved in the blood coagulation cascade like prothrombin, but could not be detected so far in hemolymph of annelids, crustaceous organisms, insects, soybean leaves, *E. coli* extract or bovine milk (Zytkovicz and Nelsestuen 1976). Accordingly, the ELISA developed in this study verified a remarkable selectivity for osteocalcin in plant background. A protein extract prepared from a classical feed mix used for the preparation of 0.1 % BMBM spiked feed samples, yielded a background signal comparable with blank and NTC. Similar results were obtained with 100 % soybean-, wheat- or corn protein extracts and a further plant based milk replacer containing 12 % citrus and 5 % cocoa shell (data not shown). The presence of beet or citrus pulp obviously does not pose a problem in contrast to test interference observed for these ingredients in a previous validation study on commercial test kits (Boix et al. 2004).

The selectivity of the assay was also excellent in fish meal background which is explainable by the pronounced sequence differences between osteocalcin from bone fish compared with terrestrial animals (Figure 1). In contrast, the observed weak but significant cross reactivity with pure pig bone or pig meal decreased the selectivity of the assay. However, only one sample of pure pork meal was available for testing so that further pork MBM materials as well as defined mixtures have to be investigated to verify this result. Due to the nature of the material a cross-contamination with bovine material could not be excluded. The identification of MBM traces derived from other species – e.g. BMBM in pork or fish meal – seems to be a most challenging issue associated with MBM targeted immunological methods which was also observed for commercial cooked meat kits. In studies on the performance on two broadly distributed dip stick tests increasingly false positive results were monitored in the presence of 5 % pork meal or animal fat or with fish meal at levels as low as 1.5 % (Fumière et al. 2009; Boix et al. 2004).

The percentage of bone fragments in two defined BMBM samples which we used in our experiments was about 54 and 80 % (w/w), which is the common range reported for
materials from rendering plants (Liu et al. 2010). The good results yielded with EDTA as a bone solvent is in line with recently published results on an improved extraction method for the detection of ruminant material. Due to an EDTA-based extraction mode the performance of a commercial dip stick test could be improved from 2 % down to a detection level of 0.1 % BMBM in feed (Karanam 2011). In contrast to our approach the method includes a concentration step of the bone fraction by sedimentation with 100 ml tetra chloroethylene (TCE) as used in sample preparation for microscopy (Regulation (EC) No 152/2009). An additional density based concentration of bone fragments is not necessary for the ELISA method described here avoiding an agent supposed to be carcinogenic (IPCS INCHEM; http://www.inchem.org/). However, an over night bone digestion step which does not allow rapid sample analysis within a few hours, was also applied in our study. To speed up the extraction method used here the procedure could possibly be optimized by hot and/or acid extraction or augmentation of the sample amount or further homogenization of coarse fractions.

The bone content might play an essential role regarding the sensitivity of the method. The question if an osteocalcin based method would also perform with samples containing < 54 % (w/w) bone or even with 100 % muscle meal remains to be clarified with appropriate materials which were not available in this study. With respect to the expectable great variance of MBM from different rendering plants it would be anyhow advisable not only to rely on one single target molecule but to apply a panel of well defined marker-proteins to capture the great diversity of animal by-products produced by the rendering industry.

Although the sensitivity of our assay 1 ng bovine OC is about one order of magnitude below commercial osteocalcin ELISA validated for pharmaceutical applications, the sensitivity with MBM looks encouraging compared with commercial ELISA test kits developed for the scope of species detection in processed meat. For these, sensitivities of 0.1, 0.5 or >=2 % MBM in feed or even a complete failure to detect legally treated MBM have been reported from
diverse validation studies (Boix et al. 2004; Karanam 2011; Meyers et al. 2007; von Holst et al. 2006; Rao and Hsieh 2007; Love and Carey-Smith 2004; Gizzi and von Holst 2004). The sensitivity of the ELISA procedure used in our study primarily might have been decreased during the mono-specific purification step retaining most potent binding IgG from the polyclonal sera. The linkage to the voluminous streptavidine molecule had no negative impact on the sensitivity compared with horseradish peroxidase labelling (data not shown) but an enhancing effect due to multiple biotinylation sites.

Conclusions

In conclusion our results demonstrate that osteocalcin is a promising tissue specific target protein for the sensitive immunological detection of meat and bone meal in compound feed or fish meal with an excellent selectivity for BMBM in these matrices. Furthermore environmental bone fragment contaminants in sugar beet epidermis, e.g. derived from rabbit, rat or mouse can be easily discriminated because of marked sequence divergences to e.g. bovine osteocalcin. The here described antibody-based osteocalcin approach might be the basis for a validated detection tool for official law enforcement of the feed ban. Moreover the general approach using epitop-specific antibodies is target-directed and might be an improvement in terms of the acceptance as national or international standard in the future. Moreover quantification is possible based on a standard curve established by commercially available pure bovine osteocalcin.

References


matrix-assisted laser desorption ionization/time-of-flight mass spectrometry and high-resolution hybrid mass spectrometry. Analytica Chimica Acta. 693: 89-99


Love JL, Carey-Smith GV. 2004. Immunoassay Kit Used to Detect the Presence of Bovine

Assessment of Two Enzyme-Linked Immunosorbent Assay Tests Marketed for Detection of
Ruminant Proteins in Finished Feed. Journal of Food Protection. 70(3): 692-699

of processed animal proteins in animal feedstuffs. International Journal of Food Science and
Technology. 39: 851-861

Preservation of the bone protein osteocalcin in dinosaurs. Geology. 20(10): 871-874

Nielsen-Marsh CM, Richards MP, Hauschka PV, Thomas-Oates JE, Trinkaus E, Pettitt PB,
Karavanic I, Poinar H, Collins MJ. 2005. Osteocalcin protein sequences of Neanderthals and
modern primates. PNAS. 102(12): 4409-4413

Nishimoto SK, Waite JH, Nishimoto M, Kriwacki RW. 2003. Structure, Activity, and
Distribution of Fish Osteocalcin. Journal of Biological Chemistry. 278(14): 11843-11848

Pérez-Calvo E, Castrillo C, Baucells MD, Guada JA. 2010. Effect of rendering on protein and
e154-e163

detection of beef and sheep content in raw and cooked meats. Meat Science. 76: 489-494

of 22 May 2001 laying down rules for the prevention, control and eradication of certain
transmissible spongiform encephalopathies. Official Journal of the European Communities.

COUNCIL of 21 October 2009 laying down health rules as regards animal by-products and
derived products not intended for human consumption and repealing Regulation (EC) No


Zytkovicz TH, Nelsestuen GL. 1976. \( \gamma \)-Carboxyglutamic Acid Distribution. Biochimica et Biophysica Acta. 444: 344-348
Figure 1. Comparison of osteocalcin sequences published for farmed animals, pets, rodents, amphibian and bone fish species. Conserved carboxylated glutamic acid residues at position 17, 21 and 24 are asterisked. Black arrows point to tryptic cleavage sites. Amino acid exchanges between Bos and other farmed animals are highlighted in bold types. The position of the sequence targeted by the detection antibody PAB OC1-9 and the N-MID-fragment (1-43) are marked by black lines. The capture antibody OC4-30 (Takara) used in the sandwich assay in this study binds to the epitope around position 17.

Figure 2. Specificity of PAB OC1-9 (polyclonal anti-osteocalcin IgG-fraction) towards native bovine bone protein in western blot analysis. M, Lane 2: Low-Range Molecular Weight Marker (3.5-40 kDa), Amersham, GE Healthcare; Lane 1: NTC (water instead protein); Lane 3: OC=Positive Control (bovine osteocalcin, 1.3 µg/slot); Lane 4: BB=bovine bone EDTA extract (7.2 µg/slot)

Figure 3. Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Sensitivity with purified bovine osteocalcin. Error bars indicate a one-fold standard deviation. Measurements were done in three replicates on three different days. Blank=PBS instead of capture; NTC= PBS instead of sample; *significant difference (p<0.05) against NTC and Blank.

Figure 4. Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Specificity with EDTA-extracts from native bone and feed components. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

Figure 5. Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Method performance with meat and bone meal extracts. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

Figure 6. Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Selectivity in mixtures containing 0.1 % bovine MBM. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.
Comparison of osteocalcin sequences published for farmed animals, pets, rodents, amphibian and bone fish species. Conserved carboxylated glutamic acid residues at position 17, 21 and 24 are asterisked. Black arrows point to tryptic cleavage sites. Amino acid exchanges between Bos and other farmed animals are highlighted in bold types. The position of the sequence targeted by the detection antibody PAB OC1-9 and the N-MID-fragment (1-43) are marked by black lines. The capture antibody OC4-30 (Takara) used in the sandwich assay in this study binds to the epitope around position 17.
Specificity of PAB OC1-9 (polyclonal anti-osteocalcin IgG-fraction) towards native bovine bone protein in western blot analysis. M, Lane 2: Low-Range Molecular Weight Marker (3.5-40 kDa), Amersham, GE Healthcare; Lane 1: NTC (water instead protein); Lane 3: OC=Positive Control (bovine osteocalcin, 1.3 µg/slot); Lane 4: BB=bovine bone EDTA extract (7.2 µg/slot)

100x106mm (150 x 150 DPI)
Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Sensitivity with purified bovine osteocalcin. Error bars indicate a one-fold standard deviation. Measurements were done in three replicates on three different days. Blank=PBS instead of capture; NTC= PBS instead of sample; *significant difference (p<0.05) against NTC and Blank.

![Graph showing A450 nm vs osteocalcin (OC) concentration per well/100μl](image)

<table>
<thead>
<tr>
<th>Standard</th>
<th>Day one Mean</th>
<th>Day one SD</th>
<th>Day two Mean</th>
<th>Day two SD</th>
<th>Day three Mean</th>
<th>Day three SD</th>
<th>Intra assay variation CV (%)</th>
<th>Inter assay variation CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.246</td>
<td>0.041</td>
<td>0.182</td>
<td>0.01</td>
<td>0.258</td>
<td>0.058</td>
<td>16.6</td>
<td>5.5</td>
</tr>
<tr>
<td>NTC</td>
<td>0.244</td>
<td>0.014</td>
<td>0.168</td>
<td>0.014</td>
<td>0.307</td>
<td>0.09</td>
<td>5.6</td>
<td>3.6</td>
</tr>
<tr>
<td>OC 0.1ng</td>
<td>0.219</td>
<td>0.014</td>
<td>0.229</td>
<td>0.004</td>
<td>0.226</td>
<td>0.031</td>
<td>5.4</td>
<td>1.5</td>
</tr>
<tr>
<td>OC 1ng</td>
<td>0.363</td>
<td>0.043</td>
<td>0.288</td>
<td>0.037</td>
<td>0.464</td>
<td>0.123</td>
<td>11.8</td>
<td>12.9</td>
</tr>
<tr>
<td>OC 10ng</td>
<td>1.267</td>
<td>0.1</td>
<td>1.125</td>
<td>0.137</td>
<td>1.369</td>
<td>0.174</td>
<td>7.9</td>
<td>11.3</td>
</tr>
<tr>
<td>OC 100ng</td>
<td>3.386</td>
<td>0.186</td>
<td>3.36</td>
<td>0.113</td>
<td>3.536</td>
<td>0.103</td>
<td>5.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>

210x143mm (150 x 150 DPI)
Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Specificity with EDTA-extracts from native bone and feed components. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

165x92mm (150 x 150 DPI)
Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Method performance with meat and bone meal extracts. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

166x90mm (150 x 150 DPI)
Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Selectivity in mixtures containing 0.1 % bovine MBM. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.
Table 1. Protein concentration of EDTA extracts used in ELISA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein concentration of the undiluted extract used per well [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMBM. 145°C</td>
<td>0.278</td>
</tr>
<tr>
<td>BMBM. 133°C</td>
<td>0.283</td>
</tr>
<tr>
<td>BMBM dog feed</td>
<td>0.124</td>
</tr>
<tr>
<td>Plant Feed 0.1% (w/w) BMBM</td>
<td>0.094</td>
</tr>
<tr>
<td>Plant Feed 0% BMBM</td>
<td>0.068</td>
</tr>
<tr>
<td>fish meal</td>
<td>0.193</td>
</tr>
<tr>
<td>pork MBM</td>
<td>0.124</td>
</tr>
<tr>
<td>sheep MBM</td>
<td>0.182</td>
</tr>
<tr>
<td>poultry meal</td>
<td>0.098</td>
</tr>
<tr>
<td>bovine bone</td>
<td>0.055</td>
</tr>
<tr>
<td>sheep bone</td>
<td>0.163</td>
</tr>
<tr>
<td>goat bone</td>
<td>0.267</td>
</tr>
<tr>
<td>pork bone</td>
<td>0.242</td>
</tr>
<tr>
<td>chicken bone</td>
<td>0.182</td>
</tr>
<tr>
<td>rabbit bone</td>
<td>0.117</td>
</tr>
<tr>
<td>horse bone</td>
<td>0.075</td>
</tr>
<tr>
<td>horse serum</td>
<td>0.878</td>
</tr>
<tr>
<td>hen´s egg</td>
<td>0.406</td>
</tr>
<tr>
<td>whole milk powder</td>
<td>0.231</td>
</tr>
</tbody>
</table>
Table 2. Selectivity in osteocalcin specific sandwich ELISA, using MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody, for hidden BMBM in pork meal in three independent measurements

<table>
<thead>
<tr>
<th>Extract</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMBM dog feed</td>
<td>3.469</td>
<td>3.352</td>
<td>3.501</td>
<td>3.441</td>
<td>0.078</td>
</tr>
<tr>
<td>Pork MBM</td>
<td>0.677</td>
<td>0.753</td>
<td>0.586</td>
<td>0.672</td>
<td>0.084</td>
</tr>
<tr>
<td>10% BMBM in pork MBM</td>
<td>0.887</td>
<td>1.116</td>
<td>1.232</td>
<td>1.078</td>
<td>0.176</td>
</tr>
<tr>
<td>1% BMBM in pork MBM</td>
<td>0.525</td>
<td>0.561</td>
<td>0.595</td>
<td>0.560</td>
<td>0.035</td>
</tr>
<tr>
<td>0.1% BMBM in pork MBM</td>
<td>0.476</td>
<td>0.524</td>
<td>0.514</td>
<td>0.505</td>
<td>0.025</td>
</tr>
</tbody>
</table>