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Immunochemical Detection of Tissue from the Central Nervous System via Myelin Proteolipid Protein: Adaptation for Food Inspection and Development of Recombinant Bivalent Fab Mini-Antibodies

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Bovine spongiform encephalopathy (BSE) is transmitted by the ingestion of central nervous system (CNS) tissue of infected animals. Food inspection must, therefore, test for the presence of CNS tissue in meat and meat products. A Western blot assay for the specific CNS tissue marker myelin proteolipid protein (PLP) was optimized with considerably reduced analysis time, solvent consumption, and detection limit (0.001% CNS tissue in minced beef). Further, a PLP-specific recombinant bivalent fragment antigen binding mini-antibody (anti-PLP Fab) was obtained from a commercial phage display library. Western blot analysis with the anti-PLP Fab selectively detected CNS tissue in minced beef with a detection limit of 0.025%. Model experiments for meat processing revealed that assay sensitivity decreased with increasing temperature and prolonged heating time. A market survey with 687 sausage samples was performed using PLP-Western blot and enzyme-linked immunosorbent assay (ELISA) for glial fibrillary acidic protein (GFAP). Five samples were tested clearly positive by both assay systems, whereas in an additional six samples, CNS tissue was detected only by GFAP ELISA and in two samples only by PLP-Western blot.

KEYWORDS: Bovine spongiform encephalopathy (BSE); central nervous system (CNS); myelin proteolipid protein (PLP); monoclonal antibody; recombinant bivalent Fab mini-antibodies; phage display library; Western blot

INTRODUCTION

Consumption of meat from cows suffering from bovine spongiform encephalopathy (BSE) (*1*) is considered the main cause for the development of the new variant of Creutzfeld-Jakob disease (vCJD) in humans (*2*). The highest concentration of the prion PrP^{sc}, which is most likely the infectious agent of BSE and vCJD, is found in the central nervous system (CNS), mainly in brain and spinal cord, whereas muscle meat, blood, other organs, or milk are regarded as safe (*1*). In order to eliminate potential risks for the consumers' health, specified risk material such as bovine CNS tissue is banned from the human food chain in the EU, Canada, the USA, and Switzerland (*3*). In some countries, such as Germany, the use of any CNS material in meat products must be declared (*4*). As a consequence, analytical methods are required to control possible CNS contamination in meat as well as the addition of CNS tissue to meat products in order to enforce the legal regulations.

Several markers have been suggested for the detection of CNS tissue in food, namely, cholesterol, the protein markers neuron-specific enolase, myelin basic protein, synaptophysin, and glial fibrillary acidic protein (GFAP), as well as brain specific fatty acids (*5*−*10*). Whereas cholesterol is usually detected by photometry, neuron-specific enolase has been analyzed by Western blot. Synaptophysin is localized by immunohistochemistry, and brain specific fatty acids are examined by gas chromatography/mass spectrometry. For routine GFAP analysis, an enzyme-linked immunosorbent assay (ELISA) is applied, but GFAP messenger RNA (mRNA) can also be tested by reverse transcription-polymerase chain reaction coupled to restriction length polymorphism analysis (*11*, *12*). Very recently, an immuno-polymerase chain reaction (PCR) assay has been described for the sensitive analysis of GFAP protein (*13*). Front-faced fluorescence spectroscopy of lipofuscin, a high molecular weight fluorescent polymer that is enriched in neuronal tissue, was tested as a screening method for contamination with CNS tissue during the slaughter process (*14*).

Myelin proteolipid protein (PLP) was recently identified as a highly specific marker for CNS tissues (*15*, *16*). PLP is a main component of central nerves contributing to about 50−55% of the total protein content of their myelin sheaths. Its full-length variant is predominantly expressed in the central nervous system, but not in peripheral nerves (*17*). Because of high hydrophobicity, PLP can be easily and selectively enriched by a simple organic solvent extraction (*15*, *18*). A polyclonal antibody was raised

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against the peptide corresponding to amino acids 109−127 of full-length PLP and successfully included in a Western blot protocol that detected CNS material in meat and meat products and on contact surfaces with high sensitivity and specificity (*15*). In addition, a dot blot assay with the polyclonal PLP antibody was developed for the rapid analysis of CNS tissue on meat and contact surfaces (*19*). A study by Hammon et al. detected the addition of CNS material in almost 10% of the tested retail sausage samples using a PLP-Western blot protocol (*20*).

An analysis method based on a polyclonal antibody, however, is disadvantageous for an official standard protocol, because batch-to-batch variations of polyclonal antibodies require revalidation. One purpose of the present study was to develop recombinant bivalent fragment antigen binding mini-antibodies (Fabs) specific for PLP that could be adopted for an official standard protocol. Furthermore, the Western blot protocols were optimized for use in food inspection.

MATERIALS AND METHODS

Reagents and Technical Equipment. All chemicals (Sigma-Aldrich, Taufkirchen, Germany) and solvents (Fisher Scientific, Schwerte, Germany) were of analytical grade. For sample homogenization, a kitchen mixer (Moulinette, Giessen, Germany) or a T18 Ultra Turrax (IKA, Staufen, Germany) was used. The extracts were dissolved using a Thermomixer (Eppendorf, Hamburg, Germany). Electrophoresis with precast gels was performed in a Mini-Protean 3 cell (Bio-Rad, Munich, Germany) or a SureLock XCell (Invitrogen, Karlsruhe, Germany), with NuPAGE bis-tris and Novex tris-glycine precast gels (Invitrogen, Karlsruhe, Germany), Ready-Gels (Bio-Rad, Munich, Germany, or SERVAgels (Serva, Heidelberg, Germany). For protein transfer, a semidry blotting device (Biometra, Goettingen, Germany) was employed. During incubation of the membranes, the solutions were agitated on a Stuart SR9 roller mixer (Bibby Scientific, Staffordshire, UK). Centrifuge tubes (Greiner, Solingen, Germany) were used for sample extraction as well as incubation of the membranes.

Generation of Recombinant Fabs against PLP. Recombinant Fabs binding to PLP were selected and generated from an antibody phage display library by a commercial provider (HuCAL GOLD library, AbD serotec, Martinsried, Germany). For this purpose, the peptide comprising the amino acids 109−127 of PLP was synthesized and coupled to bovine serum albumin (BSA) and human transferrin as described before (*21*). Two different carrier proteins were used in order to avoid the selection of antibodies with affinity toward the carrier proteins by alternating selection. Two parallel library selections on the peptide antigens were performed as described in the literature, and enriched pools were subcloned into different expression vectors (*21*). After three rounds of alternating selection on the BSA and transferrin peptide conjugates with blocking against the carrier molecules BSA and transferrin, the enriched pools of Fab genes were isolated and cloned into *Eschericia coli* expression vectors leading to the functional periplasmic expression of bivalent Fab fragments by an in-frame fusion of the antibody VH−CH1 gene segment with a self-dimerizing helix−turn−helix motif (format Fab-dHLX) (*21*) or with bacterial alkaline phosphatase (BAP) (format Fab-A) (*22*). A total of eight Fab fragments with unique sequences were isolated and purified for testing of specificity in ELISA. For this purpose, 20 μL of peptide conjugates and of other control antigens at a concentration of 5 μg/mL were coated and blocked with 5% BSA. Then crude Fab-containing supernatant obtained after cell lysis or 2 μg/mL of purified Fab, respectively, were added. Antihuman IgG $F(ab)_2$ specific AP conjugate (STAR126A, AbD Serotec, Martinsried, Germany) was diluted 1:5000, and added to the wells. Detection was performed using AttoPhos (Roche, Mannheim, Germany) and measuring fluorescence at an excitation of 440 nm ±25 nm and emission of 550 nm ±35 nm.

The selection of the eight clones, which were provided by the commercial supplier, was further tested by us for binding to full-length PLP by Western blotting as described below, with the following exceptions: The concentrations of Fab primary antibody ranged up to 10 μg/mL and the goat antihuman IgG $F(ab')_2$ secondary antibody was diluted at 1:2000. Incubation times were 18 h at room temperature and 3 h at room temperature, respectively.

Table 1. Assay Sensitivity for Heat-Treated Samples[a]

temperature/ time (°C)	polyclonal anti-PLP antibody				recombinant anti-PLP Fab	
	unprocessed	30 min	60 min	120 min	unprocessed	60 min
	0.001				0.025	
70		0.005	0.01	0.01		0.05
80		0.025	0.05	0.1		0.5
90		0.25	0.25	1		>5[b]
100		0.5	2.5	2.5		>5[b]

[a] Lowest reliably detectable concentrations of bovine brain tissue in minced beef given in [%]. [b] The highest concentration tested (5%) was not detected.

The recombinant Fabs were bivalent and contained either a BAP (Fab-A) dimerization domain followed by a Flag-His tag (clones '5691' − '5695') or a helix−turn−helix (Fab-dHLX) dimerization domain followed by a Myc-His tag (clones '5702' − '5704') at the C-terminus of the antibody heavy chain.

Samples and Sample Treatment. Minced beef purchased in a local butcher shop was used as a matrix for spiking experiments. CNS tissue (bovine brain) was provided by the Max Rubner-Institute (MRI), Kulmbach, Germany. Dilution series of a suspension of CNS tissue in tris-buffered saline (TBS, 50 mM tris-HCl, 150 mM NaCl, pH 8.0) were prepared after homogenizing bovine brain tissue in TBS in the Ultra Turrax. Aliquots of 5 g of minced beef were weighed into 50 mL centrifuge tubes and spiked with the diluted suspensions of CNS tissue.

Extracts of pure CNS tissue and of minced beef containing 5% CNS tissue were used to preselect the recombinant Fab clones with good affinity to full-length PLP. For the determination of assay sensitivity, samples with 0.001% to 0.25% CNS tissue (w/w) in minced beef were prepared. In order to study the influence of sample heating on assay sensitivity, samples with 0.005% up to 5% CNS tissue in minced beef (w/w) were prepared. Aliquots of 5 g of each of these samples were placed at the bottom of test tubes. The closed tubes were then heated in a water bath at different temperatures for the time periods as indicated in **Table 1**. Afterward, samples were cooled and kept at 4 °C until extraction.

Retail sausage samples were collected in 2007 (n = 213) and 2008 (n = 474) by the Bavarian Health and Food Safety Authority. The samples were homogenized in a kitchen mixer and stored at −20 °C until extraction. Reference material containing 0%, 0.1% (spinal cord), 0.2% (brain), 1% (brain), and 2% (brain) porcine CNS tissue was purchased from R-Biopharm (Darmstadt, Germany). These emulsion-type sausages had been heated at 80 °C for 1 h during preparation as indicated by the manufacturer.

Protein Extraction. Standard extraction of PLP was performed according to Sandmeier et al.(*15*) To adopt the protocol for food inspection, the following changes were made: Five grams of homogenized sample material were extracted with 20 mL of *n*-hexane by vigorous shaking for 1 min. The suspension was filtered (folded filters, MN 615 1/4, Macherey-Nagel, Dueren, Germany). The sample tube was rinsed with 10 mL of *n*-hexane and the solvent was filtered. The solvent was removed from the combined filtrates under reduced pressure using a rotary evaporator at 100−200 mbar and 35 °C. To remove lipids, the residue was resuspended in 10 mL diethyl ether and centrifuged at 2650*g* for 10 min at 10 °C. After discarding the supernatant, the residue was resuspended in 1.5 mL of diethyl ether and centrifuged at 2000*g* for 1 min at room temperature. The supernatant was discarded again and the residue was dried. Subsequently, the residue was dissolved in Laemmli sample buffer (125 mM tris-HCl, 140 mM sodium dodecyl sulfate (SDS), 200 mM dithiothreitol, 20% glycerol (v/v), and 0.02 mM bromphenol blue, pH 8.5) (*23*) at a concentration of 20 μg/μL using a thermomixer adjusted to 1400 rpm for 1−2 h at room temperature. The solutions were kept at 4 °C until use.

SDS−PAGE and Western Blot with Polyclonal Anti-PLP Antibody. Prior to analysis, the PLP-solutions were diluted 1:4 in Laemmli sample buffer. For analysis with the polyclonal antibody (anti-PLP rabbit serum, gift from the Institute for Biochemistry, University of Erlangen, Germany prepared according to Villmann et al.) (*16*), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting were conducted according to Sandmeier et al. (*15*) but with some modifications. Aliquots of 35 μg of protein extract per lane were loaded into a 15% polyacrylamide gel and separated at 140 V for 75 min in

running buffer (25 mM tris-HCl, 200 mM glycine, 0.1% SDS). The proteins were transferred to a nitrocellulose membrane (Protran BA 85, pore size 0.45 μm; Whatman, Dassel, Germany) at 150 mA for 45 min with a semidry blotter using transfer buffer (25 mM tris-HCl, 150 mM glycine, 10% methanol). Membranes were stained with Ponceau solution (0.2% Ponceau S in 3% trichloroacetic acid) for 2−3 min to examine proper protein transfer and then washed with TBS (50 mM tris-HCl, 150 mM NaCl, pH 7.4). All following incubation steps were accomplished at room temperature placing the membrane in a centrifuge tube on a roller mixer, except when noted otherwise. The membranes were washed with TBS after each incubation step. First, the membrane was incubated with blocking buffer (5% skim milk powder, 0.5% Triton X-100 in TBS) for 60 min. Subsequently, the blot was incubated with the anti-PLP antiserum diluted 1:200 in dilution buffer (3% BSA, 0.5% Triton X-100, TBS) for 18 h at 4 °C, followed by incubation for 2 h with an alkaline phosphatase (AP) conjugated secondary antibody (goat antirabbit IgG-AP, Dianova, Hamburg, Germany), diluted 1:5000 in dilution buffer. The antibody-binding was visualized with 5-bromo-4-chloroindolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT) as a precipitating colorimetric substrate. Stock solutions containing 50 mg/mL BCIP in dimethylformamide (DMF) and 50 mg/mL NBT in 70% DMF were prepared and stored at −20 °C. The substrate solution was prepared by adding 35 μL of BCIP solution and 60 μL of NBT solution to 10 mL substrate buffer (100 mM tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) immediately before use. The enzymatic reaction was stopped after 6−10 min by replacing the substrate solution with stop solution (20 mM tris-HCl, 20 mM ethylenediaminetetraacetate (EDTA), pH 8.0). Finally, the blot was dried for storage and scanned for documentation purposes on a flat bed scanner. A band visible at the position of PLP was considered a positive signal and compared to a negative control (minced beef without CNS tissue) where no signal was observed.

Western blot analysis with precast gels was performed analogously, except for the use of 3-(*N*-morpholino)propanesulfonic acid (MOPS-)SDS as running buffer for SDS−PAGE with bis-tris gels according to the manufacturer's instruction.

SDS−PAGE and Western Blot with PLP-Specific Recombinant Fabs. The adoption of the newly developed recombinant Fab '5702' (AbD Serotec, Martinsried, Germany) required further modifications of the system. Optimized conditions employ NuPage 12% bis-tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane) gels and MOPS-SDS as running buffer (both Invitrogen, Karlsruhe, Germany) for SDS−PAGE. The blots were incubated with 10 μg/mL primary antibody, followed by incubation with the enzyme-labeled secondary antibody (goat antihuman IgG $F(ab')_2$-AP, AbD Serotec, Martinsried, Germany) in a dilution of 1:2000. To improve washing results, TBS containing 0.5% Triton X-100 was used before substrate incubation. The enzyme reaction was carried out for 20−30 min.

Tyramide Signal Amplification (TSA). As an amplification step, TSA was introduced into the Western blot protocol as described before (*24*). This method requires a BAP-labeled Fab. Therefore, the Fab '5691' was used for this particular experiment, because it detected PLP in the Western blot and is labeled with BAP. Briefly, after incubation of the membrane with blocking buffer, the blot was incubated with primary antibody Fab '5691'- BAP (2 μg/mL in dilution buffer) for 2 h, followed by incubation with horseradish peroxidase anti-BAP secondary antibody (AbD Serotec, Martinsried, Germany, 1:5000 in dilution buffer) for 1 h. The biotinylated tyramide solution was diluted 1:500 in TBS containing 0.01% H_2O_2 and the blot was irrigated therein for 5 min. After extensive washing with 30 mL of TBS containing 0.5% Triton X-100 three times, the membranes were incubated with streptavidin-AP (AbD Serotec, Martinsried, Germany, 1:5000 in dilution buffer) for 30 min. Extensive washing was repeated before the bands were visualized by the BCIP/NBT substrate system. Biotinylated tyramine was prepared as described by Kerstens et al. (*25*).

Immunochemical Detection of GFAP by ELISA. Retail sausage samples were tested for supplementation with CNS tissue by the Ridascreen Risk Material ELISA kit (R-Biopharm, Darmstadt, Germany) as per the manufacturer's instructions. The ELISA kit is based on the immunochemical detection of GFAP as a marker protein for CNS tissue in processed meat. According to the manufacturer's instructions, a sample is considered positive if OD_{450} [sample] > 2 × OD_{450} [matrix blank = standard 1]. The lowest value of the standard curve corresponds to 0.2% CNS tissue.

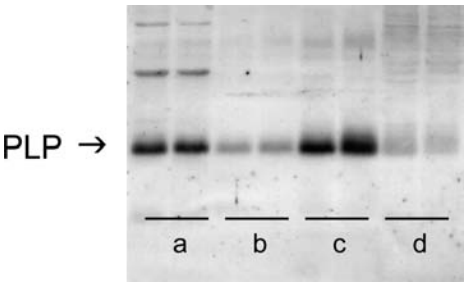

Figure 1. Western blot analysis of commercial sausage samples containing CNS tissue. Samples of four different sausage types (a: salami; b: liver sausage; c: emulsion-type sausage; d: fermented sausage) were extracted following standard procedure (left lanes) and following the optimized extraction protocol (right lanes respectively).

RESULTS AND DISCUSSION

Optimization of PLP-Western Blot for Food Inspection. Western blot analysis of PLP proved to be a reliable method for the detection of CNS tissue in meat and meat products. However, the method developed by Sandmeier et al. (*15*) was not ideal for food inspection, as routine protocols should be not only reliable but also fast and easy to perform. Therefore, an improved, less time-consuming sample workup protocol has been developed. It considerably reduces the number of extraction steps and the consumption of solvents for the preparation of PLP extracts. Furthermore, the Ultra Turrax is no longer necessary for sample homogenization, thus limiting the risk of cross contaminations. Both extraction protocols were applied to test four different commercial sausages containing detectable amounts of CNS tissues. **Figure 1** shows that the simplified workup protocol produces Western blot results that are comparable to those of the established extended workup.

The protocol for Western blotting was optimized in a similar way to reduce analysis time and the number of required working steps. Thus, it was even possible to improve the assay sensitivity by a factor of 25 compared to the established method as described by Sandmeier et al., reaching a detection limit of 0.001% CNS tissue in minced beef (*15*).

Finally, casting gels is often difficult to include into routine food monitoring. Therefore, several precast gels of different suppliers were tested. The application of precast gels, however, generally led to a remarkable loss of assay performance. Acceptable results were obtained using a 16% tris-glycine gel, leading to a slight loss of sensitivity by a factor of 5 (data not shown).

Development of a Recombinant Bivalent Fab Anti-PLP Mini-Antibody from a Commercial Phage Display Library. The use of a polyclonal antiserum for immunochemical methods can hamper food inspection by batch-to-batch variations of antibody affinity and quality. Thus, the development of a standardized official method is difficult. As far as we know, only one monoclonal antibody against PLP is commercially available (*15*). Unfortunately, it cannot be applied for the detection of contaminations with CNS tissue due to a cross reactivity with DM-20, a splice variant of PLP naturally present in peripheral nerves (*15*). To overcome this problem, a recombinant antibody against PLP was prepared using the amino acids 109−127 of the primary PLP sequence as the target hapten. It has been shown that this sequence provides antibodies with optimal selectivity for CNS tissue (*16*). For this purpose, Fab fragments, which bind selectively to the hapten peptide, were selected from a commercial phage display library (*26*). Positive clones were then expressed as bivalent Fab mini-antibodies. Thus, eight recombinant Fabs were obtained that recognized the target peptide hapten

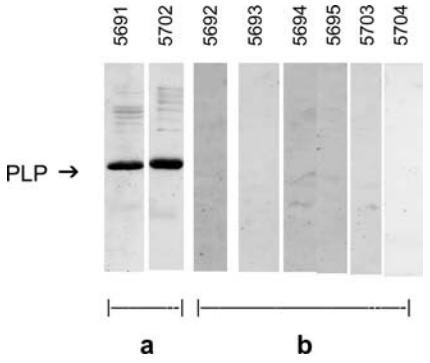

Figure 2. Western blot analysis of CNS tissue using preselected recombinant Fabs with affinity to the hapten peptide. Each lane was loaded with 35 μg of protein extract of (a) minced beef containing 5% bovine CNS tissue and (b) 100% CNS tissue. The recombinant Fab clones '5691' − '5695' and '5702' − '5704' were applied as primary antibody for the detection of PLP.

('5691' − '5695' and '5702' − '5704'). To select the most suitable recombinant Fabs, PLP-extracts of minced beef supplemented with 5% CNS tissue were analyzed by Western blot using an enzyme-labeled secondary antibody (**Figure 2**). Two of the preselected Fabs, '5691' and '5702', produced a strong signal for PLP, whereas no bands could be detected with the remaining clones '5692', '5693', '5694', '5695', '5703', and '5704'. Further tests with PLP-extractions from pure CNS material confirmed that the latter were not able to recognize the epitope within the complete protein at all.

In addition, the clones '5691' − '5695' were expressed including the enzyme BAP that can directly detect the bound antibodies in the Western blot, thus avoiding the secondary antibody. Direct detection of PLP via the fusion-enzyme BAP bound to Fab '5691' produced bands of similar intensity as those obtained by an AP-labeled secondary antibody (data not shown).

The two active recombinant Fabs were compared employing extracts from the dilution series of CNS tissue. The tests revealed that the Fab '5702' yielded a 2-fold higher sensitivity. Fab '5702' was, therefore, selected for further analysis (data not shown).

On the basis of a phage display library, recombinant antibody fragments are produced completely in vitro. This technique is, therefore, faster and less expensive than the conventional preparation of monoclonal antibodies. Although they were used in food monitoring before, for example, for the analysis of mycotoxins or pesticides, recombinant antibody fragments from synthetic phage display libraries are still seldom applied in this field (*27*−*30*).

Comparison of Assay Sensitivity Using the Polyclonal Anti-PLP Antibody and the Recombinant Anti-PLP Fabs. The systematical variation of parameters determined optimal assay conditions for the recombinant antibody. The best results were obtained applying 10 μg/mL of the primary antibody '5702' for 18 h and the secondary anti-F(ab')$_2$ antibody in a dilution of 1:2000 for 2 h.

Under these optimized conditions, extracts from the dilution series of CNS tissue were tested to determine the assay sensitivity. The lowest CNS tissue concentration detectable in minced beef amounted to 0.025%, whereas 0.001% CNS tissue in minced beef could be reliably detected with the polyclonal antibody under optimized analysis conditions (**Figure 3**). It can be concluded that the recombinant antibody detects PLP in raw meat with good sensitivity.

To our knowledge, the correlation between the level of contamination with CNS tissue and a potential infectivity is not established. Therefore, the legal regulations do not give maximum residue limits of specified risk material or CNS tissue. Instead, maximal sensitivity must be endeavored in order to exclude the presence of CNS tissue in food.

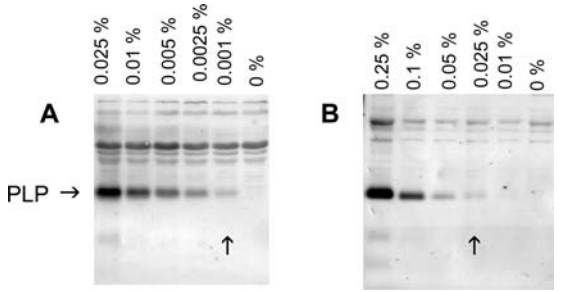

Figure 3. CNS tissue dilution series to determine the sensitivity of the Western blot with the polyclonal anti-PLP antibody and the recombinant anti-PLP Fab. Bovine CNS tissue was added to minced beef in concentrations of 0.001% up to 0.25% before extraction. Unspiked minced beef served as a negative control. Extracts were analyzed using a polyclonal anti-PLP serum (A) and the recombinant PLP-specific Fab '5702' (B). The position of the specific band for PLP at about 29 kDa and the lowest detected concentrations are marked by arrows. Note that the initial concentration in panel B is 10 times higher than the initial concentration in panel A.

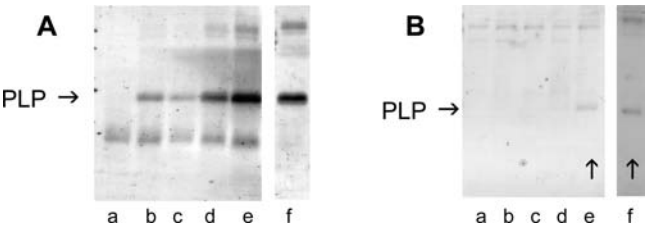

Figure 4. Analysis of standardized samples spiked with CNS tissue using the polyclonal anti-PLP antibody (A) and the recombinant PLP-specific Fab '5702' (B). Reference material of emulsion-type sausage containing 0% (a), 0.1% (b), 0.2% (c), 1% (d), 2% (e) porcine CNS tissue was extracted. In addition, a retail sample of similar type containing a large amount of CNS tissue was analyzed (f).

TSA, a technology to enhance signal intensity (*24*), was tested to improve the assay sensitivity. The method is based on the deposition of biotinylated tyramine at the location of antibody-binding by a peroxidase-labeled secondary antibody. The precipitate can then be visualized by AP-labeled streptavidin and BCIP/NBT as substrate. Although the signal could be amplified by TSA, the increase in background led to an unspecific signal in the area of PLP (data not shown). This approach was therefore not pursued.

Analysis of Reference Sample Material. Standardized sample material containing 0%, 0.1% (spinal cord), 0.2% (brain), 1% (brain), and 2% (brain) porcine CNS tissue in an emulsion-type sausage was analyzed with both the polyclonal anti-PLP antibody and the recombinant anti-PLP Fab. In addition, an emulsion-type retail sample with high CNS tissue contamination was tested. The recombinant anti-PLP Fab could not detect PLP in the samples supplemented with less than 2% CNS tissue, whereas the polyclonal antibody detected PLP in all spiked samples (**Figure 4**). The retail sample produced a strong signal when the polyclonal antibody was used, but only a band of weak intensity with the recombinant anti-PLP Fab.

Although the recombinant anti-PLP Fab shows good sensitivity for the detection of CNS tissue in unprocessed material, the sensitivity is not sufficient for the analysis of low CNS tissue concentrations in meat products.

Influence of Thermal Sample Treatment on Assay Sensitivity. A model system simulating common conditions during sausage preparation was used to determine the influence of sample heating on the assay sensitivity. Dilution series of CNS tissue in minced beef were heated in a water bath for 30, 60, and 120 min at 70, 80, 90, and 100 °C before extraction. The PLP extracts were then analyzed by Western blot with the polyclonal anti-PLP antibody. The samples heated for 60 min were additionally examined with the recombinant anti-PLP Fab.

Table 1 shows the detection limits after heat treatment. In this model, an acceptable loss of sensitivity can be observed for the polyclonal antibody. This result is in line with a previous study that reported some decrease of signal intensity after a meat sample spiked with 3.5% CNS tissue had been heated to 75 and 95 °C (*15*). In contrast, the assay based on the recombinant anti-PLP Fab considerably lost sensitivity when it was applied to heat-treated samples. The limit of detection shifted to a range unsuitable for the analysis of low concentrated samples.

Contaminations of meat products by CNS tissue can be caused by different sources. Brain tissue can be used as an ingredient to improve the technological sausage properties or for adulteration. Furthermore, CNS tissue can be a component of advanced meat recovery products, or splattering of spinal cord during the slaughtering process can occur (*6*, *20*, *31*, *32*). The recombinant anti-PLP Fabs were able to detect CNS tissue in raw meat with good sensitivity. Therefore, it is expected that the selected anti-PLP Fabs can be applied for the analysis of CNS tissue in advanced recovery meat products or as a part of a dot blot assay to detect splattering of CNS tissue during slaughtering (*19*). In contrast to polyclonal antibodies, recombinant Fabs have the advantage that they are available in constant quality in a theoretically unlimited quantity. Thus, they can be included in an official standard protocol without the requirement of batch-to-batch revalidation. In comparison to conventional monoclonal antibodies, the generation of recombinant Fabs is faster, less expensive, and accomplished completely in vitro. However, field studies are required to confirm the assumption that the newly developed anti-PLP Fabs can be used to screen raw meat for the presence of CNS tissue. On the other hand, heat treatment of the sample considerably decreased the affinity of the antigen to the recombinant anti-PLP Fabs. As a consequence, the assay sensitivity of the Western blot using anti-PLP Fabs was much lower compared to the Western blot using the polyclonal anti-PLP antibody when processed meat was investigated. Therefore, anti-PLP Fabs could not be used to screen for the presence of CNS tissue in heat-treated meat products. Signal enhancement strategies, such as tyramide signal amplification, were not successful when the complex meat matrix was analyzed. The reason for the observed decrease of antibody affinity is not known. It could be tested, however, in future studies, if other libraries or other antibody formats will be able to generate recombinant antibodies with a higher affinity toward the heat treated PLP-epitope.

Analysis of Contaminations of Retail Products with CNS Tissue. The test system for CNS tissue contaminations based on the polyclonal anti-PLP antibody was included in the sausage monitoring plan of the Bavarian Health and Food Safety Authority. A survey study on the prevalence of CNS tissue in 687 sausage samples and meat products from the Bavarian retail was carried out applying a commercial ELISA kit for GFAP. Fifty-one of these samples were reanalyzed by Western blot using the polyclonal anti-PLP antibody. The selected samples were either representative for the whole collection or had evoked a positive signal. The test results are summarized in **Table 2**. Among the 51 samples, five samples were tested clearly positive by both assay systems, whereas for six samples, positive results were obtained only by the GFAP ELISA, and for additional two samples only by the PLP-Western blot. Addition of CNS tissue had not been declared for any of the tested samples.

Table 2. Analysis of CNS Tissue in Retail Samples of Sausages and Meat Products Examined under the Regular Control Plan of the Bavarian Health and Food Safety Authority by GFAP ELISA and Anti-PLP Western Blot[a]

product declaration	results [% CNS tissue] GFAP ELISA	results Western blot	sample type
salami	>col	++	raw sausage
salami	0.33/n.d.	−	raw sausage
salami	0.20/n.d.	−	raw sausage
teewurst	0.24/0.27	+	raw sausage
mettwurst	>col/n.d.	−	raw sausage
mettwurst	n.d.	+	raw sausage
salami	>col	−	raw sausage
mettwurst	>col	−	raw sausage
teewurst	>col	++	raw sausage
landjaeger	>col/n.d.	−	raw sausage
wiener	n.d.	+	emulsion-type sausage
bruehwurst	>col	−	emulsion-type sausage sterilized
liver sausage	>col/n.d.	−	precooked sausage
liver sausage	>col/n.d.	−	precooked sausage
liver sausage	>col/n.d.	−	precooked sausage
lyoner	>col	−	emulsion-type sausage
liver sausage	0.22/ > col	−	precooked sausage
liver sausage	>col/>col	−	precooked sausage
gelbwurst	0.26/0.22	+++	emulsion-type sausage
gelbwurst	n.d.	+	emulsion-type sausage
gelbwurst	n.d./>col	−	emulsion-type sausage
gelbwurst	0.22/0.23	++	emulsion-type sausage
liver sausage	>col/n.d.	−	precooked sausage
liver sausage	>col/n.d.	−	precooked sausage
presskopf	>col/n.d.	−	precooked sausage

[a] 26 samples yielded clear negative results in both test systems: raw meat (1), cooked meat (1), raw sausage (8), emulsion-type sausage (9), emulsion-type sausage sterilized (1), pre-cooked sausage (6).

n.d., the value was below the manufacturer's cutoff level of 2 × OD_{450} [matrix blank]; > col; the value was higher than the manufacturer's cutoff level, but lower than the lowest standard of 0.2% CNS tissue. −, no signal; +, weak signal; ++, strong signal; +++, very strong signal in the Western blot.

Since the true composition of the samples is not known, it can only be speculated why the results from PLP-Western blot and GFAP ELISA diverged for eight samples. The reading of four of the samples, which were tested positive by GFAP ELISA, but negative by PLP-Western blot, was around the cutoff level of the ELISA. Particularly in the range between the cutoff level and the lowest standard, a lack of precision of the GFAP ELISA was observed: In 11 samples, signals slightly above the cutoff level in the ELISA were not reproducible after retesting (**Table 2**). Thus, this imprecision can result in false positive results from the GFAP ELISA. The diverging results may also be caused by differences in the detection limits of both methods. The sensitivity of the PLP-Western blot is remarkably higher compared to the GFAP ELISA (*15*). However, the present study also showed a drop in the sensitivity of the Western blot, when heated samples were analyzed. Differences in the sensitivity of the GFAP ELISA between raw and processed samples have not been studied systematically. It can be speculated that the decrease of sensitivity is lower, leading to an actual higher sensitivity of the GFAP ELISA compared to the PLP-Western blot, when processed meat is analyzed. Finally, false positive results could also be generated by cross reactivity of the antibody with tissues from organs other than CNS. Whereas PLP is very selectively expressed in CNS, cross reaction of the GFAP antibody, for example, with peripheral nerve tissue, has been described in the past (*6*, *16*).

It is not known if the CNS tissue detected in the meat products was specified risk material, which is banned from the food chain. In the EU, specified risk material is defined mainly as CNS tissue from cow, sheep, and goats of certain age and geographical origin (*3*). To date, no analytical method is available that differentiates specified risk material from other CNS tissue, so that the analysis of specified risk material is still limited to the detection of CNS tissue. In some countries, such as Germany, the use of any animal brain and spinal cord is not accepted for the production of meat products, unless it has been declared on the label (*4*).

In a previous study on sausages from the Bavarian market collected in 2005, nearly 10% of the samples were tested positive (*20*). In contrast, CNS tissue could be detected in less than 2% of the samples collected in 2007/2008 for the present study, indicating that the use of CNS tissue or raw material contaminated with CNS tissue for the preparation of meat products has decreased considerably.

The present study shows that the generation of recombinant bivalent Fab mini-antibodies from a commercial phage display library can be a cheap and fast alternative to produce antibodies for immunochemical food analysis. The recombinant anti-PLP Fabs obtained here, however, showed lower affinity toward the heat-treated epitope compared to the polyclonal antibodies, excluding their application for the analysis of CNS tissue in processed meat. The results of a field study that analyzed 687 retail products by PLP-Western blot point out that the undeclared addition of CNS tissue to sausages is rare but still remains an issue for food inspection.

ABBREVIATIONS USED

BSE, bovine spongiform encephalopathy; vCJD, variant Creutzfeld-Jakob disease; CNS, central nervous system; GFAP, glial fibrillary acidic protein; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PLP, myelin proteolipid protein; Fabs, bivalent fragment antigen binding mini-antibodies; BSA, bovine serum albumine; BAP, bacterial alkaline phosphatase; TBS, tris-buffered saline; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AP, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloroindolyl phosphate/nitroblue tetrazolium chloride; DMF, dimethylformamide; MOPS, (3-(*N*-morpholino)propanesulfonic acid); TSA, tyramide signal amplification.

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