JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Myelin Proteolipid Protein (PLP) as a Marker Antigen of Central Nervous System Contaminations for Routine Food Control

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Spreading transmissible spongiform encephalopathies (TSE) have been widely attributed to transmission by ingestion of mammalian central nervous system (CNS) tissue. Reliable exclusion of this epidemiological important route of transmission relies on an effective surveillance of food contamination. Here, myelin proteolipid protein (PLP) is identified as a specific and largely heat-resistant marker for detection of food contaminations by CNS tissue. PLP is a component of oligodendritic glial sheaths of neuronal processes that is specifically expressed in the CNS. A highly selective polyclonal antibody was developed directed against an epitope present in the full-length PLP protein, but absent from the developmentally regulated splice variant DM-20. In combination with a hydrophobic extraction of PLP from tissue samples, the antibody reliably detected PLP from spinal cord, cerebellum, and cortex of different mammalian species. Consistent with earlier reports on PLP expression, no cross-reactivity was observed with peripheral nerve or extraneural tissue, except for a very faint signal obtained with heart. When applied to an artificial CNS contamination present in sausages, the antibody reliably detected a low concentration (1%) of the contaminant. Application of heat, as used during conventional sausage manufacturing, led to a predominant alteration of arginine residues in the PLP protein and a partial loss of immunoreactivity. In contrast, a stretch of hydrophilic amino acids$^{112-122}$ proved to be heat-resistant, preserving the immunogenicity of this PLP epitope during heating. Taken together, the excellent CNS specificity of PLP immunodetection and the presence of a heat-resistant epitope have permitted the development of a highly sensitive immunoassay for CNS contamination in routine food control.

KEYWORDS: Myelin proteolipid protein; CNS; food contamination; polyclonal antibody

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of neurogenerative diseases, comprising scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), feline spongiform encephalopathy, transmissible mink encephalopathy, and chronic wasting disease. In humans, TSEs include variant Creutzfeld−Jakob disease (vCJD), fatal familial insomnia, kuru, and Gerstmann−Sträussler−Scheinker syndrome (*1*, *2*). There is strong epidemiological evidence linking the transmission of BSE and vCJD disease to the ingestion of infectious material from cattle and sheep (*3*−*5*). The infectious material appears to consist of prion protein (PrP), which had been converted to its detrimental conformer PrP^{sc} (*6*). In affected animals, PrP^{sc} predominantly accumulates in the central nervous system (CNS), that is, the brain and spinal cord. In contrast, the contribution of other tissues, for exampe, retina or lymphoid tissues, to the transmission of BSE is not fully understood (*7*−*9*). According to our current understanding, there is no evidence for the transmission of spongiform encephalopathies to the human consumer via skeletal muscle of infected animals (*10*).

One of the primary means of consumer protection is, therefore, to diagnose BSE in cattle that are slaughtered for human consumption. However, a drawback of the currently available BSE tests lies in their relatively low sensitivity, in addition to their inability to detect infection of living animals (*11*). Commissioned by the U.S. Department of Agriculture, the Harvard Center of Risk Analysis identified the inclusion of high-risk tissue as one of three potential pathways for human exposure to infectious BSE agents (*12*). In particular, consumption of brain and spinal cord from cattle has been associated with a high risk of disease transmission. As a secondary means of consumer protection, exclusion of risk tissues and contaminations from human consumption is indispensable. In a similar

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10.1021/jf0707278 CCC: $37.00
Published on Web 07/13/2007

way, the Scientific Steering Committee of the European Union (EU) and the World Health Organization (WHO) have attributed a high potential of infectivity to bovine brain and spinal cord.

Potential routes of ingestion of CNS tissue include either direct consumption of brain and spinal cord or contamination of meat products through advanced meat recovery or by bone-in cuts of meat, such as T-bone steak (*12*). In addition, other tissues classified into this highest category are mainly included because of their potential contamination with brain or spinal cord (*13*). As a consequence, many countries, including the EU countries (*14*), the United States (*15*), Canada (*16*), and Switzerland (*17*), have consequently banned the presence of CNS tissue in beef and beef products.

To control these novel requirements in consumer protection, highly specific and sensitive assay systems are required to detect and unequivocally confirm the presence of CNS tissue in meat and meat products. Currently, established tests for CNS contamination frequently rely on neural enolase 2 (NSE) or glial fibrillary acidic protein (GFAP) as marker proteins, but may be limited by false-positive results as both antigens are not exclusively expressed in CNS (*18*). In this study, we identified myelin proteolipid protein (PLP1 or PLP) as a novel marker protein of food contamination by CNS tissue. PLP is a component of glial sheaths that is specifically expressed by oligodendrocytes in the brain and spinal cord. Mutations of the human gene PLP1 result in the dysmyelinating Pelizaeus−Merzbacher disease disorder (*19*, *20*). Together with myelin basic protein (MBP), small myelin-associated protein (S-MAG), and mitogen-activated protein kinase (MAPK), PLP assembles into protein complexes in the axonal oligodendroglial membrane (*21*, *22*). In addition to the CNS, the PLP gene is also transcribed in the peripheral nervous system (PNS), but with low abundance (*23*, *24*). However, the isoform prevailing in PNS is DM-20, an alternative pre-mRNA splice variant resulting from the exclusion of exon 3b (*25*).

Following an extensive analysis of gene expression databanks, we confirmed PLP as a highly specific CNS marker protein. As a tool of immunological analysis, a polyclonal antibody was developed that specifically recognized the CNS-specific full-length variant of PLP, even after extended application of heat as is common in food processing. In combination with a hydrophobic extraction procedure, this antibody was applied to the selective immunodetection of BSE risk material in meat products.

MATERIALS AND METHODS

Databank Search. To define appropriate candidate proteins that are exclusively expressed in CNS specific tissue, databases of the NCBI (National Center of Biotechnology Information, National Library of Medicine, National Institute of Health, USA, Bethesda, MD; //www.ncbi.nlm.nih.gov/) and the European Bioinformatics Institute (EBI, Cambridge, U.K.; //www.ebi.ac.uk) were used. Detailed analysis of expression profiles identified the myelin proteolipid protein (PLP1) as highly CNS specific. Candidate proteins were further verified using SAGE (www.ncbi.nlm.nih.gov/SAGE). SAGE is a highly efficient technique that allows global analysis for gene expression (*26*). The technique produces 9−10 base sequences ("tags") that identify one or more mRNAs. Frequencies of sequenced, concatenated tags reflect the cellular abundance of the corresponding transcript. Sequencing of many thousands of gene-specific tags leads to an expression pattern of a specific tissue.

Peptide Generation and Immunization. The PLP sequence was analyzed for antigenicity. On the basis of its flexibility and hydrophilicity, a highly conserved epitope in the middle of the protein sequence was chosen for antibody generation (^{109}CGKGLSATVTGGQKGRG-SR^{127}). The peptide was synthesized by a solid-phase peptide synthesis performed on a 9050 PlusPepSynthesizer (Millipore) using Fmoc-PAL-PEG-PS-resin to construct a peptide-amide. The integrity and purity of the lyophilized crude peptide were verified by reversed-phase high-performance liquid chromatography (HPLC) analysis and mass-assisted laser desorption ionization−time of flight mass spectrometry (MALDI-TOF-MS). Without further purification, the crude peptide was coupled to keyhole limpet hemocyanin (KLH) via the heterobifunctional cross-linker sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (Sulfo-SMCC). This protocol is most effective for high-density coupling of epitope peptides to carrier proteins (*27*). To generate polyclonal antibodies, rabbits (Chinchilla Bastards) were immunized with 400 μg of peptide conjugate per boost. After analysis of the preimmune serum, rabbits were immunized three times at days 0, 14, and 28. The first test blood was taken 5.5 weeks after the first boost.

Peptide Chromatography and ELISA. Following drying of 200 μL of PLP peptide (1 μg/μL), the peptide was dissolved in 50 μL of 0.2 M pyridine. Trypsin digest was carried out overnight using 1 μg of trypsin at room temperature. Chromatographic conditions were controlled with a Merck-Hitachi L-6200 system supplemented with a diode array UV detector (L-7450A). The diode array detector signal was recorded on a computer using D-7000 HSM software (Merck): flow rate, 0.75 mL/min; buffer, 0.1% trifluoroacetic acid (TFA); gradient, 0−50% acetonitrile in 30 min; column, Beckman ODS Ultrasphere (5 μm, 4.6 × 250 mm); detection, UV at 206 nm. The enzyme-linked immunosorbent assay (ELISA) was carried out using 100 μL of each fraction for coating on Nunc Immuno modules. PLP peptide dissolved in PBS was used as a positive control (5 and 1 μg/μL; 500, 100, 50, and 10 ng/μL). The peptide used for commercial antibody generation served as a negative control. Blocking was done with Tris-buffered saline (TBS)/0.1% Tween-20/1% BSA for 1 h at room temperature. Afterward, plates were incubated with the PLP-antibody K16 (dilution 1:500) for an additional hour. For detection of the antigenic signal, HRP-coupled secondary antibody (dilution 1:10.000) and TMB (Pierce) as a substrate were used.

Immunoaffinity Chromatography. For capturing antigens from biological fluids on the basis of their affinity to the corresponding antibody prior to MALDI-TOF-MS, an Immunocapturing Kit 100 MB-IAC Prot G (Bruker Daltonics) was used. The commercial PLP-antibody (Biotrend, Köln, Germany) and the generated anti-PLP antibodies from polyclonal sera were immobilized on protein G coupled magnetic particles. Following the manufacturer's instructions, the antigen either subjected to heat treatment (6 h, 95 °C) or untreated was captured by immobilized antibody and eluted for matrix preparation.

MALDI-TOF-MS. An aliquot of the eluted sample (0.5 mL) was mixed with 0.5 mL of CCA-matrix (3-hydroxy-α-cyanocinnamic-acid; saturated solution in 30% acetonitrile/0.1% TFA), dotted onto a steel target, and air-dried. MALDI-TOF analysis was performed on an Autoflex (Bruker Daltonics) in the linear mode. Desorption of the samples was carried out using a nitrogen laser (337 nm), and the probes were accelerated with 19 kV after a delay of 100 ns. For one sample spectrum, 250 individual spectra of the respective probes were performed and averaged. A peptide standard mix served as an external calibration.

Hydrophobic Protein Extraction. Homogenized sample material (2.5−5 g) was extracted three times with at least 4 mL of *n*-hexane/1 g of sample by repeated homogenization using an UltraTurrax. Removal of solvent was achieved with a rotary evaporator. To remove lipids, probes were resuspended in 30 mL of diethyl ether and centrifuged at 3500 rpm for 10 min at 8 °C. The supernatant was discarded. The lipid removal step was repeated once. The resulting off-white pellet was dried and stored at −21 °C.

Membrane Preparation from Tissue. Briefly, 1 g of CNS tissue (spinal cord and cortex of either mouse or rat origin) was homogenized in buffer H [25 mM KH_2PO_4, 25 mM K_2HPO_4, pH 7.4; protease inhibitors (Roche), 10 μM PMSF, 2.5 mM EGTA pH 8.0, 2.5 mM EDTA pH 8.0 KOH] using a glass pestle and an Ultraturrax. After centrifugation at 35000*g* and 4 °C for 20 min, pellet homogenization was repeated and centrifugation was again performed using the same conditions. The pellet was resuspended in small volumes of buffer B [10 mM KH_2PO_4, 10 mM K_2HPO_4, pH 7.4; protease inhibitors (Roche), 10 μM PMSF, 2.5 mM EGTA/K^+ pH 8.0, 2.5 mM EDTA/K^+ pH 8.0,

Table 1. Database Search for Appropriate CNS Marker: Tissue Expression

CNS-specific candidate protein	expression pattern
PLP (proteolipid protein)	CNS: hypothalamus, hippocampus, whole brain, adult brain, fetal brain, dorsal root ganglia, cerebellum, right hemisphere, amygdala, medulla, adrenal gland, subthalamic nucleus, multiple sclerosis lesions sensory organs: iris, cochlea, fetal eyes tumors: melanotic melanoma, melanocyte, high-grade transitional cell tumor, mammary adenocarcinoma cell line, germ cell tumor, astrocytoma, nervous-tumor, parathyroid tumor, endometrium, medulloblastoma, nervous-normal glioblastoma, oligodendroglioma others: testis, heart, prostate, amnion, skin, bone marrow, sympathetic trunk
NSE (neural enolase 2)	CNS: hippocampus, whole brain, head-neck, adult brain, fetal brain, hypothalamus, hypothalamus sensory organs: retina, optic nerve, retina foveal and macular, fetal eyes, lens, eye anterior segment, pineal gland, trabecular meshwork tumors: epidermal tumor, melanotic melanoma, lymphoma, retinoblastoma, glioblastoma with EGFR amplification, neuroblastoma, renal cell tumor, B-cell, chronic lymphotic leukemia, squamous cell carcinoma, pituitary glioblastoma, glioblastoma, melanocyte, hypernephroma others: colon, amnion, ovary, heart, blood, lung, spleen, purified pancreatic islet, breast, testis, skin, bone marrow, sympathetic trunk
GFAP (glial fibrillary acidic protein)	CNS: brain, head-neck, fetal brain, optic nerve, hippocampus, hypothalamus, brain hemisphere, dorsal root ganglia, cerebellum, pituitary gland, dura mater, pineal body, multiple sclerosis lesions sensory organs: fetal eyes, human retina, lens, eye anterior segment, retina foveal and macular, pineal gland, eye anterior segment tumors: glioblastoma with EGFR amplification, nervous-tumor, glioblastoma without EGFR amplification, kidney-tumor, oligodendroglioma, astrocytoma, germ cell tumor, anaplastic oligodendroglioma others: lung, testis, colon, amnion, placenta, larynx, bone marrow

200 mM KCl]. Before storage at −80 °C probes were shock frozen in liquid nitrogen. Protein concentrations were determined using the standard Bradford assay, and 40 μg of protein was loaded on a SDS-gel (*28*).

Sodium Dodecyl Sulfate−Polyacrylaminde Gel Electrophoresis (SDS-PAGE) and Immunodetection. For SDS-PAGE lipophilic extracts or membrane preparations from different tissues were dissolved in 2× sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerin, 4% SDS, 0.1% bromphenolblue, 2% β-mercaptoethanol). Tissue preparations were denatured for 20 min at 56 °C and lipophilic extracts for 25 min at 75 °C. For protein separation, 15% polyacrylamide gels were used. Proteins were transferred onto a nitrocellulose membrane (Protan BA85, Schleicher & Schüll) at 150 mA for 1 h using a Western blotting device (Biometra).

After 1 h of blocking with TBB [50 mM Tris-HCl, 150 mM NaCl, 0.5% (w/v) Triton X-100, 3% bovine serum albumin, pH 8.0], the nitrocellulose membrane was incubated with the preimmune serum and the specific polyclonal rabbit serum (1:50 to 1:5000 in TBB) for 1 h at room temperature. Following three washing steps for 10 min in TBS, TWS [50 mM Tris-HCl, 150 mM NaCl, 0.5% (w/v) Triton X-100, pH 8.0], and TBB the nitrocellulose membrane was incubated with the secondary antibody (goat anti-rabbit IgG coupled to the fluorochrome Cy5, 1:200 in TBB) for 45 min at room temperature. After washing in TBS, TWS, and TBS for 10 min each, the blot was analyzed using the STORM860 Phosphoimager (Molecular Dynamics).

Quantitative Analysis of Species Specificity. Following immunodetection on the Phosphoimager, evaluation of the specific PLP band was performed using Image Quant 5.0 (Molecular Dynamics). A rectangular area of defined size was used in each gel lane to calculate the average of absolute fluorescent intensity. For the determination of the detection limit and quantification limit, 12 blank values (porcine peripheral nerves or skeletal muscle) were analyzed as described before. The detection limit was calculated as $y_{blank} + 3.3 \times SD_{blank}$, and the quantification limit was calculated as $y_{blank} + 10 \times SD_{blank}$ (*29*).

RESULTS

Databank Search for Protein Markers of CNS Contamination. For experimental evaluation of reliable markers of CNS contamination in food, the following criteria were defined: (i) CNS-specific expression; (ii) association of the antigen with a particulate rather than soluble protein fraction; (iii) presence of heat-stable, immunogenic determinants; (iv) favorably, species specificity. Using database analyses, we attempted to identify a protein marker that most likely matched these properties.

To assess the relative expression of a particular gene in a given tissue, two bioinformatic tools are frequently used: (i) expressed sequence tags (ESTs) and (ii) serial analysis of gene expression (SAGE). ESTs are short, about 300−500 bp, single-pass sequence reads from cDNA. They represent a snapshot of genes expressed in a given tissue and/or at a given developmental stage. Determination of gene expression levels by SAGE relies on the assessment of sequence tags, about 9 base pairs in size, that are located at a specific site within a gene transcript. By coupling both technologies, it has become possible to simultaneously follow changes in expression levels for a large number of genes.

The relative tissue expression of a marker gene was assessed by the ratio of the corresponding gene ESTs in a particular tissue versus those in another tissue. This ratio obtained was normalized to the number of total ESTs in the pool and represents the selectivity of gene expression. Our expression profile analysis confirmed three candidate proteins: (1) proteolipid protein (PLP), (2) neural enolase 2 (NSE), and (3) glial fibrillary acidic protein (GFAP) are highly enriched in CNS tissue. By bioinformatic analysis, however, NSE was found to be less stringently expressed in brain (8.7/10.000; $n = 920560$), as its transcript tags were also detected in heart (0.22/10.000; $n = 88178$), lung (0.84/10.000; $n = 344147$), colon (0.3/10.000; $n = 198415$), PNS (2.99/10.000; $n = 16691$), and skin (0.48/10.000; $n = 186189$) (**Table 1**). Likewise, tags indicative of GFAP were also apparent in the brain (34.0/10.000; $n = 920560$), colon (0.9/10.000; $n = 198415$), larynx (10.8/10.000; $n = 29363$), and blood (1.5/10.000; $n = 127672$) (**Table 1**). Although PLP expression was stringently confined to the CNS (brain, 64.1/10.000; $n = 920560$), a minor expression was also detectable in heart (0.34/10.000; $n = 88178$) and skin (2.9/10.000; $n = 186189$). For PLP expression in the heart, the ratio gene EST/total EST was much lower than the corresponding value for CNS. PLP expression in thymus was not included because of low complexity of the database for that tissue. Expression in

Table 2. SAGE Analysis of CNS-Specific Candidate Antigens

candidate protein	tags (*n*)	most reliable tag	mRNA source sequences (*n*)	tag counts/ total sequence tags	matching unigene clusters (*n*)	name of unigene clusters
GFAP	1899	ACTTTGTCCC	227	682/120431	3	*GFAP* glial fibrillary acidic protein *H3F3B H3* histone family *NFATC2IP* nuclear factor of activated T-cells
NSE	1200	CCACGTTCCA	196	246/102359	2	*ENO2* enolase 2 (gamma, neuronal) *KIAA1181* endoplasmatic reticulum golgi intermediate compartment 32 kDa protein
PLP	2312	CATACATACACACATATAT	121	88/41773	1	*PLP1* proteolipidprotein (Pelizaeus–Merzbacher disease, spastic paraplegia 2)

tumor tissue was excluded from this analysis, due to the degeneration of gene expression in these cell lines and the fact that tumor tissues are not admitted for nutritional use.

A more comprehensive bioinformatic assessment of expression of the three candidate genes PLP, NSE, and GFAP was obtained by analysis of different SAGE libraries, where transcript analysis relies on a small, yet informative sequence tag (**Table 2**). Using this approach, the highest number of tags was found with PLP (2312 tags), consistent with a high level of gene expression. Whereas some of the short SAGE tags represent multiple genes, our most reliable tag for PLP matched to a single unigene cluster (**Table 2**). In contrast, equivocal results were obtained for tags representing NSE and GFAP, as they matched at least two genes. Thus, proteolipid protein was identified as a highly specific marker of CNS expression.

The sequence alignment of PLP orthologues from different species showed that the protein primary structures are highly homologous among vertebrates (**Figure 1**). In mammals, primary structures show a high degree of conservation (up to 99%), but the avian sequence from chick revealed a sparse number of amino acid substitutions clustering around a basic region near position 120 (**Figure 1**).

Epitope Selection and Generation of Polyclonal Antibodies. For immunodetection of PLP, commercial antibodies are available that we successfully employed to analyze tissues that were not subjected to prior food processing (*30*). However, the commercial antibody used failed to recognize PLP present in food after conventional sausage manufacturing (*30*). In addition to its instability, the antibody was not able to distinguish between PLP from mammalian and avian species, as the antigenic epitope resides in the far C-terminal portion of the PLP sequence (^{270}CGRGTKF^{276}) (**Figure 1**). On the basis of its high degree of flexibility and hydrophilicity, we chose another epitope situated in the middle of the protein sequence for the generation of a new high-affinity polyclonal antibody: ^{109}CGKGLSATVTGGQKGRGSR^{127} (**Figure 1**). Besides showing the highest potential immunogenicity within the PLP primary sequence, this motif is encoded by exon 3, which is partly absent in the DM-20 protein variant (*25*). In addition, this motif also harbors a cluster of amino acid exchanges characteristic for avian PLP (**Figure 1**). This epitope thus offered the chance of generating antibodies that may discriminate between mammalian and avian CNS contamination as well as DM-20. To this end, a synthetic peptide was coupled to KLH and used to immunize rabbits. Following four boosts, polyclonal rabbit serum K16 recognized a band of 29 kDa, highly specific for PLP in membrane preparations from mouse CNS (**Figure 2**). In contrast, PLP reactivity was absent in liver tissue serving as negative control, consistant with our database analysis. No cross-immunoreactivity was observed for DM-20 at 20 kDa. In all of the CNS samples analyzed, a faint band was apparent at an appropriate molecular mass of 50 kDa, indicative of PLP aggregates (*21*). In the classic preparation protocol, PLP protein was selectively enriched by hydrophobic extraction of CNS tissue (*31*). Here, we combined *n*-hexane extraction with a delipidation step with diethyl ether. Using PLP-enriched brain extracts from pig, we carried out a dilution series of the polyclonal serum K16. Although hydrophobic proteins such as PLP generally are extremely poor antigens (*32*), we were able to reach an antibody titer of 1:1000 with the rabbit serum K16 (**Figure 3**).

PLP as Suitable Target Antigen of CNS Contamination. Specified tissues from different animal species were dissected, and their purity and identity were checked histologically. When subjected to Western blot analysis, hydrophobic extracts from native CNS tissue, that is, brain and spinal cord, showed a strong immunoreactivity for PLP (**Figure 4A**). With hydrophobic extracts from non-CNS tissues including peripheral nerve, skin, lung, liver, kidney, parotid gland, and skeletal muscle, no significant immunoreactivity for PLP was detectable (**Figure 4A**) except for a very faint signal obtained with heart, consistent with earlier reports on PLP expression. Moreover, when sausage tissue was complemented with 1% native brain tissue, the presence of PLP was reliably detectable (**Figure 4A**). RT-PCR of RNA isolated from skin tissue confirmed the absence of full-length PLP observed in the immunoassay (**Figure 4B**).

Heat Stability of the Antigenic Epitope. The antigenicity of the chosen epitope was tested using heat treatment resembling the situation during food processing. An aqueous solution of the peptide was heat treated at temperatures of 37, 50, 72, and 95 °C for 6 h. After subjection to immune precipitation with antibody K14 and subsequent MALDI-TOF-MS, the antigenicity decreased compared to the untreated peptide (**Figure 5A**,**D**), but remained clearly detectable. As evident from mass spectra, the peptide is self-dimerizing, with a monomeric mass of 1817 Da compared to a dimeric mass of 3637 Da (**Figure 5A**,**B**). Following heat treatment, smaller masses appeared with peaks at 1769 and 1785 Da, corresponding to the treated monomer, and at 3574 Da corresponding to the dimer. The unmodified monomer mass peak of 1817 Da decreased to about 25% of its original size following heat treatment at 96 °C. In contrast, smaller masses significantly increased, with signals at 1785 and 1769 Da (**Figure 5B**,**E**). During heat treatment, the presumptive dimer peptide at 3637 Da was more stable than the monomer, even following a temperature step of 95 °C for 6 h. Concomitantly, the smaller dimer peak of 3574 Da increased to about 57% of the initial dimer signal (**Figure 5E**). Analyzing the mass

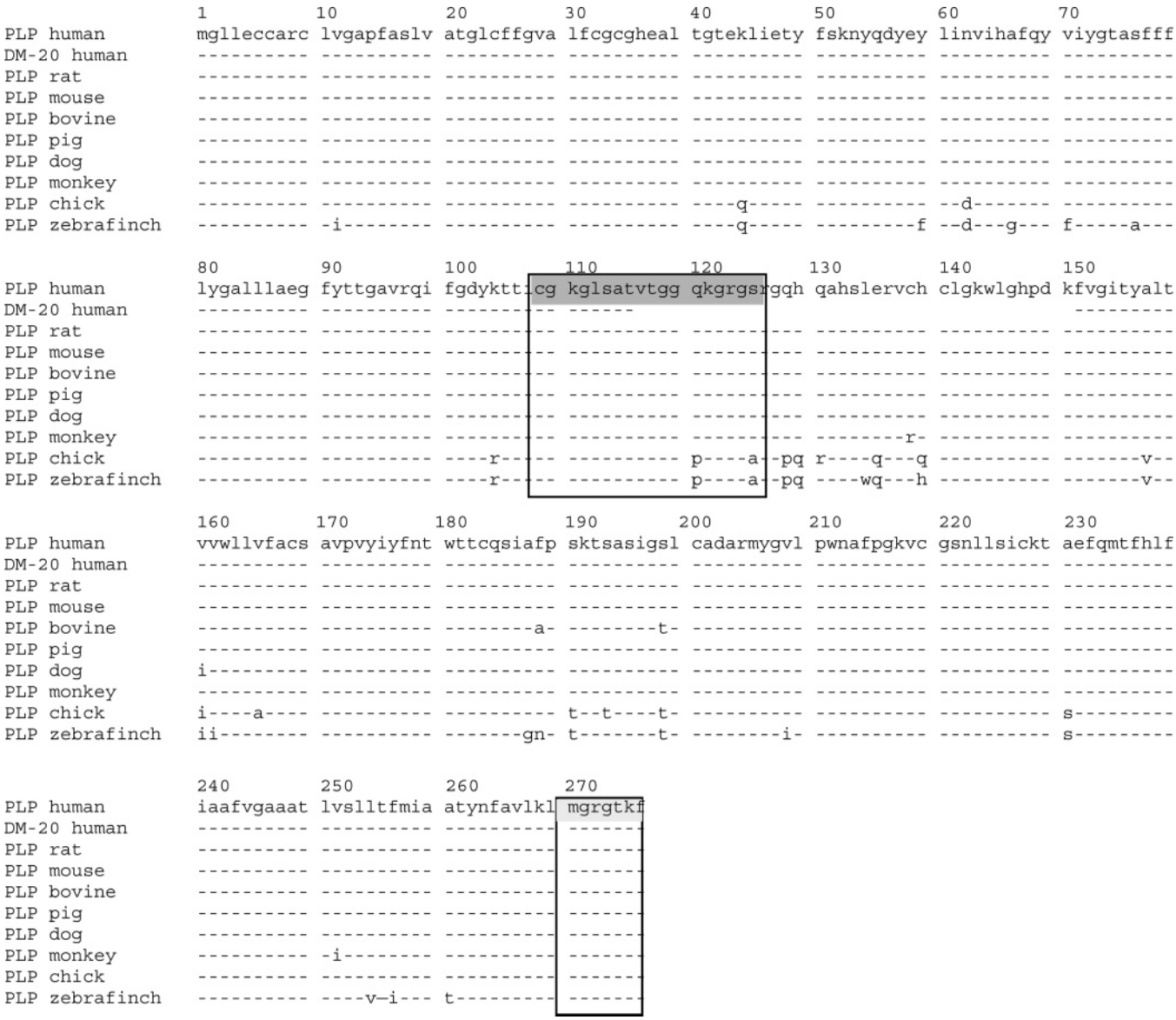

Figure 1. Alignment of PLP amino acid sequences from different species. Amino acid substitutions among species are shown with appropriate letters. Only chick PLP shows >6% differences compared to human. All other mammalian sequences are identical except bovine with two amino acid exchanges. Human DM-20 is an alternatively spliced isoform of human PLP that lacks aa$^{115-150}$ in the second intracellular loop of the protein corresponding to exon 3B. The epitope of the commercially available antibody is in the C-terminal portion of the protein (light gray box). A different epitope was chosen for generating new high-affinity antibodies (dark gray box).

differences, we speculated that the appearance of smaller mass peaks was due to a modification of positively charged residues within the antigenic epitope. The mass differences induced by heat treatment could account for a variety of peptide modifications, including deamination of arginine residues or formation of ornithine similar to the urea cycle. Similar experiments were performed with the peptide serving as antigen for the commercially available antibody. By heat treatment at 95 °C for 6 h, the antigenic peptide (767 Da) was completely converted to 733 Da (**Figure 5C**). Following heat treatment, the peptide did not bind to protein G coupled to magnetic beads, accounting for a complete loss of its antigenicity (data not shown). This indicated that the antigenic epitope selected for the commercial antibody was highly sensitive to heat treatment, consistent with a lack of PLP detection in food following heat processing.

Furthermore, the PLP peptide was digested with trypsin for mapping of the antigenic epitope. The HPLC elution profile yielded significant signals for fractions A, B, and C (**Figure 6A**). When the HPLC fractions were subjected to an ELISA using antibody K16, fractions B and C still produced significant antigenicity (**Figure 6B**). Calculations of the amino acid composition showed that the peptide present in fraction B (corresponding to fraction 19) contained amino acid positions 109−122, whereas fraction C (= fraction 20) contained amino acid positions 112−122 (**Table 3**). Thus, the narrowest constriction of the heat-stable antigenic epitope could be assigned to amino acid positions 112−122 (GLSATVTGGQK).

Species Specificity of the Novel PLP Antibody. The polyclonal serum was used to compare PLP expression in various mammalian brain preparations (pig, cow, and horse). On the basis of the relative fluorescence intensity standardized to cow brain, a clear signal was observed for all PLP extracts from mammals (**Figure 7**). The amino acid sequences of PLP orthologues from different species are highly conserved among vertebrate species, with an identity of up to 99% in mammals. In contrast, the avian sequences, that is, chick and zebrafinch

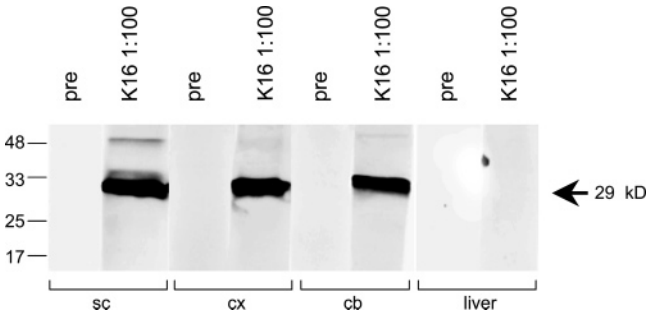

Figure 2. Polyclonal immune serum of rabbit K16 (dilution 1:100) was tested on different CNS preparations. For testing the serum a SDS-gel was loaded with membrane preparations from spinal cord (sc), cortex (cx), cerebellum (cb), and liver of mouse tissue and blotted onto nitrocellulose. Pre represents preimmune serum of the boosted animal K16. No band is observed at the appropriate molecular mass of PLP (29 kDa) in all lanes incubated with preimmune serum. Lanes loaded with CNS tissue preparations show a very strong band for PLP at 29 kDa recognized by the polyclonal K16 serum (1:100). Membrane preparation from liver was loaded as negative control.

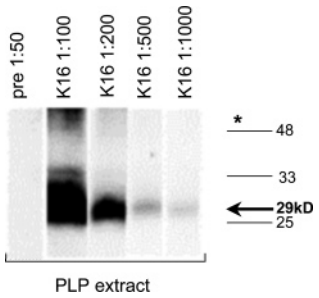

Figure 3. Dilution series for antigenicity of the polyclonal rabbit serum. Each lane was loaded with 8 μg of PLP extract from pig brain. The specific band for PLP is seen at the appropriate molecular mass of 29 kDa (dilutions shown 1:100 to 1:1000). The asterisk (*) marks a signal in the higher molecular mass range at about 50 kDa, most likely representing formation of unsolved aggregates vanishing at a dilution of 1:200 of the polyclonal serum. Pre stands for preimmune serum of the same rabbit used in a 1:50 dilution.

as contained in the genomic date bases, reveal a sparse number of amino acid substitutions clustering around the antigenic epitope chosen for antibody generation (**Figure 1**). To test for species specificity of the polyclonal serum, PLP extracts (8 μg/lane) from avian CNS samples (turkey, chicken, and duck) were subjected to semiquantitative immunodetection on a Phosphoimager. For the avian samples, fluorescence intensity was low; minced meat served as a negative control (**Figure 7**). The antigenic epitope present in the mammalian sequences differs from the avian orthologue in four amino acid positions (Q120P, S125A, Q128P, H129Q) that apparently define major immunogenic determinants allowing discrimination between mammalian and avian CNS sources (**Figure 7**).

DISCUSSION

Immunological tests for contamination of meat and meat products by CNS tissue rely on the selectivity and sensitivity of antigen detection. Here, we take advantage of the selective CNS expression of full-length PLP to develop a highly sensitive immunological probe for the detection of CNS antigen present as a contaminant in meat and meat products. A specific polyclonal antibody was raised against full-length PLP that preferentially recognized CNS contaminations of mammalian origin. In combination with solvent extraction (*31*) of the highly hydrophobic PLP from tissue samples, this antibody probe yielded a highly sensitive and reliable immunoassay.

A protein appropriate as a marker of even low amounts of CNS contaminations in products determined for nutritional use is expected to be (i) highly and (ii) specifically expressed in the target tissue, (iii) of high antigenicity, and (iv) resistant to the extraction procedure used in the final assay. To define an ample marker antigen that is both highly and selectively expressed in CNS, we used bioinformatic tools that reduced the number of appropriate candidates to PLP, NSE, and GFAP. Indeed, NSE and GFAP (*33*−*35*) have previously been used to detect CNS contaminations in meat and meat products. For PLP, bioinformatic SAGE analysis revealed a high level of expression in the CNS, and the most reliable tag was mapped to an informative single unigene cluster. Taken together, bioinformatic transcription analysis characterized PLP as a powerful candidate for establishing an assay for the detection of CNS contaminations. Established tests for CNS contamination relying on NSE, that is, the variant neural enolase 2, or GFAP as marker proteins share some problems of unspecific cross-reactivity with lung tissue, in addition to the expression of these markers in peripheral nerve tissue (*35*, *36*). As a result, combined GFAP and NSE tests may produce false-positive results as they fail to distinguish between central and peripheral nervous tissue. This limits the applicability of both proteins for routine food control, as liver and peripheral nerve tissue are common and legal ingredients of meat products.

In contrast to the other markers currently in use, its highly CNS specific gene expression characterizes PLP as an extremely reliable marker. Additional tissue specificity of PLP distribution is achieved by tissue-specific splicing of the pre-mRNA transcribed from the PLP gene: DM-20 is an alternatively spliced isoform that lacks 35 amino acids encoded by exon 3 and displays an apparent molecular mass of 20 kDa (*22*, *37*). PNS and CNS differ in protein contents of the PLP splice variant: PLP prevails in CNS (90%), whereas DM-20 predominates in PNS (*23*). DM-20 is also the major isoform of PLP in thymus (*38*). To exclude any antibody reactivity with the protein variant DM-20, the antigenic epitope used here was selectively located within the alternatively spliced exon 3, indicative of the CNS-specific full-length protein PLP. Indeed, the prediction of tissue specificity was fully confirmed by our studies at the protein level. Moreover, PLP belongs to the rare group of extremely hydrophobic proteins that, upon solvent extraction, accumulate in the lipophilic phase (*31*). To further increase the specificity of the assay, hydrophobic extraction of PLP followed by a delipidation step was introduced as an additional step of tissue preparation. No PLP signal was obtained for peripheral nerve tissue, consistent with the low expression level of PLP in the peripheral nervous system. Taken together, the polyclonal antiserum generated here against PLP was highly specific for CNS antigens and did not cross-react with extracts from peripheral nerve tissue, skin, liver, lung, or kidney, except for a very faint signal observed in hydrophobic extracts from heart tissue. Furthermore, the polyclonal antibody K16 generated here reliably detected 1% native brain tissue present in meat and meat products.

Although PLP is selectively expressed in CNS, its use as a marker of species origin of CNS tissue is limited by the high degree of conservation of its primary protein structure among vertebrates. Still, the antigenic epitope chosen here for the

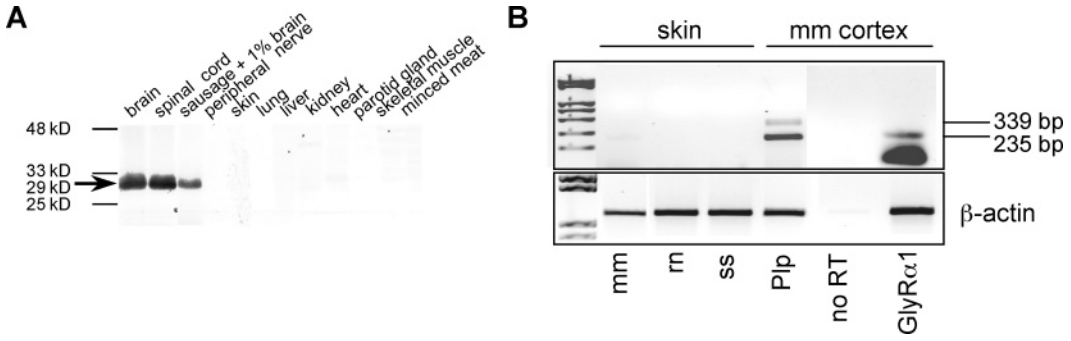

Figure 4. PLP protein and transcript detection in various tissues. (**A**) Detection of the 29 kDa isoform of PLP by the newly generated polyclonal rabbit serum (1:200) in PLP extracts (8 μg/lane) from various porcine tissues: brain, spinal cord, sausage containing 1% brain, peripheral nerve, skin, lung, liver, kidney, heart, parotid gland, skeletal muscle, negative control (minced meat). Samples were run on a 14% PAA-gel and blotted on a nitrocellulose membrane. PLP was detected by incubation with polyclonal anti-PLP rabbit serum and a peroxidase-labeled secondary antibody. (**B**) Analysis of PLP and DM20 transcripts in skin isolated from mouse (mm, *mus musculsus*), rat (rn, *rattus norvegicus*), and pork (ss, *sus scrofa*) compared to cortex isolated from mice. PLP transcripts refer to a PCR amplimer of 339 bp; DM20 transcripts are smaller (235 bp). As a positive control the glycine receptor α1 subunit was used; "no RT" refers to reverse transcription without the RT-enzyme; β-actin was used as housekeeping gene.

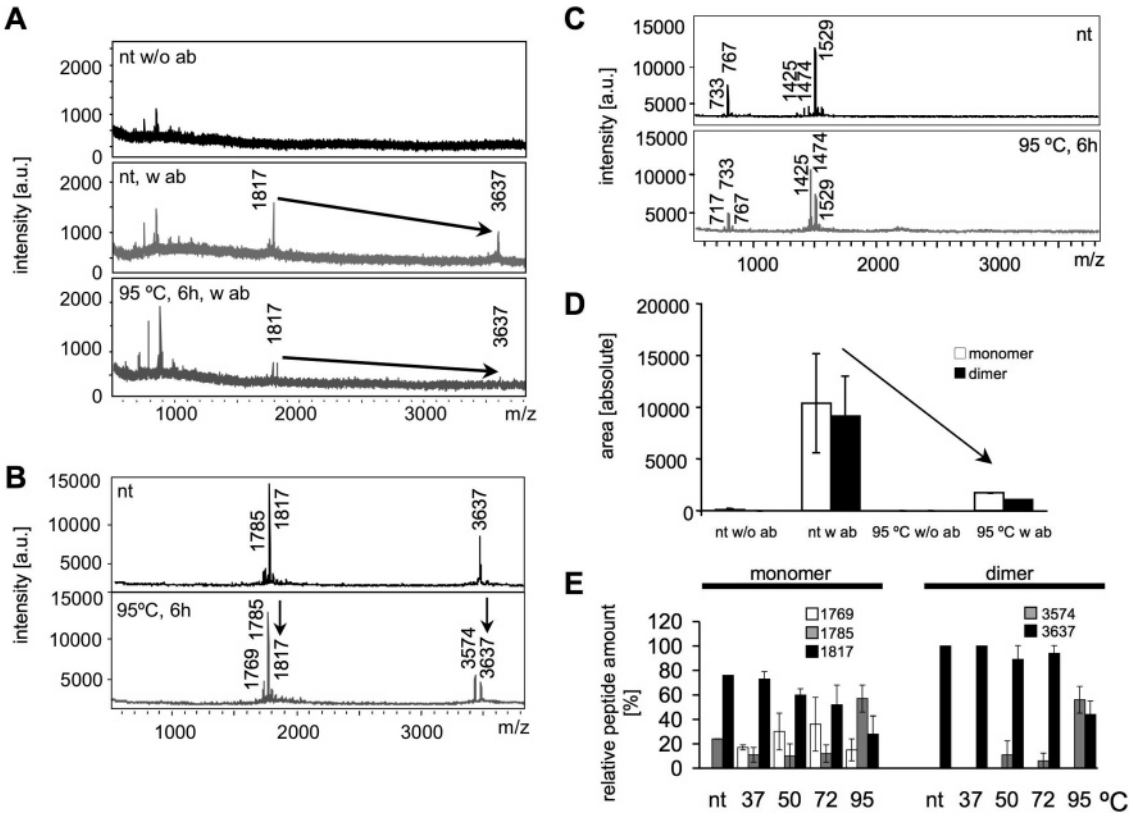

Figure 5. Stability of the antigenic PLP peptide. Following heat treatment of the peptide and several time points, MALDI-TOF-MS was used to calculate the stability of the antigenic epitope. Note that the peptide is self-dimerizing; data are shown for the monomer (1817 Da) as well as for the dimer (3637 Da). (**A**, **B**) Representative spectra of nontreated (nt) or treated peptide (95 °C for 6 h) eluted for matrix preparation from protein G coupled magnetic particles preadsorbed with (w) or without (w/o) anti-PLP antibodies (ab) from the polyclonal serum K16. Note that the monomer peak and the dimer peak are decreased following heat treatment, summarized quantitatively in (**D**) and (**E**). (**C**) Representative spectra of treated (lower spectrum) against untreated peptide (nt) (upper panel) used for commercial antibody generation. (**D**) Quantitative analysis of various spectra comparing treated and untreated peptide ^{109}CGKGLSATV-TGGQKGRGSR^{127} used for antibody K16 generation bound to protein G coupled magnetic beads presabsorbed with K16. (**E**) Bar diagram representing relative peptide amount following a 6 h treatment at different temperatures (nt, nontreated; 37, 50, 72, and 95 °C). The amount of peptide used was set to 100%. The areas below various mass peaks were calculated relative to the amount of peptide used (= 100%).

generation of a PLP-specific antibody differed between mammalian and avian species. As a result, the polyclonal antiserum K16 significantly differed in affinity for CNS extracts from the mammalian and avian species tested. Whereas a positive signal would preferentially account for a mammalian CNS contamination, further tests would be required to reliably distinguish between species. Combined ELISA tests for GFAP and NSE used for routine food control display some problems as they generate false-positive results with raw poultry tissue (*35*). So far, two different approaches have been used to overcome species problems: (i) Using gas chromatography coupled to mass spectrometry (GC-MS) analysis, it was possible to distinguish between pig, cow, and sheep brain up to sensitivities of 0.05% for brain and 0.01% for CNS material (*39*). (ii) In

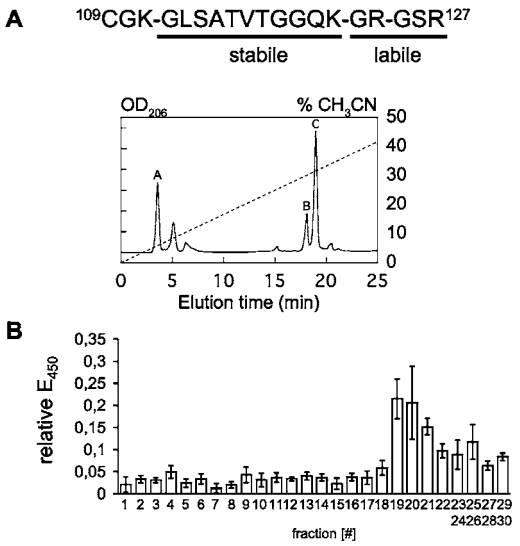

Figure 6. Digestion of the PLP peptide for antigenic epitope detection. The PLP peptide was digested using trypsin. Epitope fractions were analyzed by ELISA. (**A**) HPLC elution profile following trypsin digest. Fragments in fraction A included the first three amino acids (109–111) and the last four amino acids (123–127); fraction B corresponded to amino acids 109–122 and fraction C to amino acids 112–122; amino acid composition of the PLP peptide is shown above, see also **Table 3** (A corresponds to fraction 4, $A = 4$; $B = 19$; $C = 20$). (**B**) Quantitative test for antigenicity of all HPLC fractions. Still, fractions B and C show a clearly detectable antigenic signal compared to A and other unmarked fractions. Error bars refer to SEM values from three independent experiments.

Table 3. Antigenicity of the Trypsin-Digested PLP Peptide

fraction	relative peptide amount	sequence	ASA rests/peptide determined	ASA rests/peptide expected
20	1	112–122	Gly 3.0 Arg <0.03 Lys 0.9	Gly 3 Arg 0 Lys 1
19	0.7	109–122	Gly 3.8 Arg <0.09 Lys 1.8	Gly 3 Arg 0 Lys 2
18	0.1		Gly 2.9 Arg <0.3 Lys 1.1	
4	2 × 0.9	109–111 123–127	Ser 0.9 Gly 3.1 Arg 1.9 Lys 1.0	Ser 1 Gly 3 Arg 2 Lys 1

contrast, Lange et al. (*40*) developed RT-PCR approaches relying on GFAP and MBP transcripts as CNS markers in meat and meat products. However, although the selective detection of both markers succeeded with tissues of different mammalian origins, the instability of the mRNA species analyzed represents a major disadvantage of this approach.

The anti-PLP antibody K16 generated here not only displayed a high degree of specificity and sensitivity, but its epitope also showed a high stability. The commercially available anti-PLP

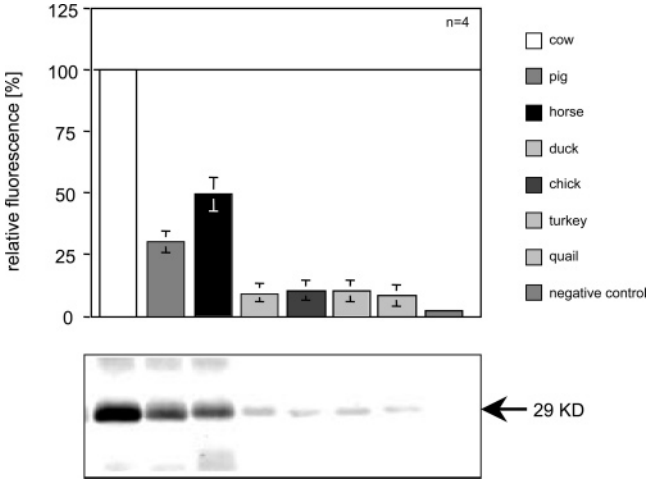

Figure 7. CNS samples of several different species were analyzed for PLP expression. The upper bar diagram represents a semiquantitative analysis of the specific PLP band at 29 kDa using Image Quant 5.0 (Molecular Dynamics). A rectangular area of defined size was used in each gel lane to calculate the average of fluorescent intensity ± standard error of the mean). For determination of the relative fluorescence intensity the average of the absolute fluorescence intensities from four independently prepared probes ($n = 4$) was used. The fluorescence intensity determined for cow brain was set to 100%. The lower panel shows a representative Western blot stained for PLP with the polyclonal antibody K16 taken for semiquantitative analysis.

antibody failed to detect PLP in processed meat products, but the polyclonal antibody K16 detected CNS tissue present in food even after conventional sausage manufacturing. In a parallel study, the reliable detection limit was below 0.1% (*30*). On the basis of a comparison of antigenic peptide epitopes, we hypothesize that the single occurrence of positively charged residues in positions ^{272}R and ^{275}K in the far C-terminal segment of the PLP sequence (^{270}CGRGTKF^{276}) may underlie the instability of this antigenic epitope following nitrite treatment or boiling of food. We propose that modification of the central amino acid ^{272}R would result in loss of antigenicity of the peptide epitope. This is consistent with a partial loss of immunoreactivity following heat treatment of PLP (*30*) and the arginine-containing antigenic peptide used here, whereas the tryptic fragment covering amino acid positions 112−122 proved to be sufficiently heat-resistant.

Despite the species barrier, BSE prions have most likely been transmitted to humans, in young adults, from infected BSE cattle. These catastrophic cases of vCJD disease show a clinical course different from sporadic Creuzfeldt−Jakob disease cases (*3*, *4*) and are thought to result from consumption of products contaminated with tissue from infected animals with BSE. Again, potential routes for ingestion of CNS tissue include either direct consumption of brain and spinal cord tissue or contamination of meat products through advanced meat recovery or by certain bone-in cuts of meat, such as T-bone steak. In addition to control of human food, the exclusion of infection routes of animals by feed requires a tight supervision control system. The anti-PLP antibody described here may provide a basis for an immunoassay applicable to routine feed control. In summary, the excellent CNS specificity and high sensitivity of the newly generated anti-PLP antibody combined with its ability to reliably detect mammalian CNS contaminations make PLP a specific CNS marker protein in routine food control.

ACKNOWLEDGMENT

We thank Drs. Katrin Schiebel and Heinrich Sticht for helpful discussions and critical reading of the manuscript. We also thank Renate Fäcke-Kühnhauser and Rosa Weber for excellent technical assistance.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of July 13, 2007, contained an incorrect version of Figure 4. This has been corrected with the posting of July 19, 2007.

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Received for review March 13, 2007. Revised manuscript received May 25, 2007. Accepted June 1, 2007. This work was supported by the Bayerischer Forschungsverbund Prionen "forprion" and DFG.

JF0707278