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RESEARCH ARTICLE

Immunological one-step determination of the central nervous system indicator proteins, neuron-specific enolase and glial fibrillary acidic protein, in meat products

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The determination of specific marker proteins is important in the prevention of infections and transmission of disease. Several diagnostic assays have been developed but these are mostly restricted to the detection of single antigens. Thus there is a need for multiplex detection assays for the simultaneous analysis of several disease indicators. Consumer protection against the transmission of prion diseases is ensured by the removal of specified risk material from cattle meat during slaughtering and this is regulated by law. The investigation reported here describes a one-step determination on immunoblots of the simultaneous detection of two indicator proteins, neuron-specific enolase and glial fibrillary acidic protein, in tissues of the central nervous system. Although the two proteins run in polyacrylamide gels with similar molecular masses the indicators are differentiated by immunological reactions followed by visualizing the different coloured specific protein bands which develop. The enolase exhibits a brown band, whereas the glial fibrillary acidic protein is red. Immunoblotting has proved to be a suitable assay for multiplex analysis of marker proteins possessing similar molecular weights and is therefore a suitable tool for application in food, veterinary and medical facilities.

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1 Introduction

The protection against the transmission of diseases increases in importance with detection of markers and indicator proteins in food and medical products. Prion diseases originating from cattle infected with agents of the bovine spongiform encephalopathy (BSE) can be transmitted to humans by consuming the meat and developing the variant Creutzfeldt–Jakob disease [1, 2]. To guard consumers against infection with prions, potentially specific risk materials (SRM), in which the infectivity titres of BSE prions are most likely the highest, such as central nervous system (CNS) tissues including the spinal cord in meat products and raw meat in particular, have to be removed from the food chain during the slaughtering process. Thus bovine CNS tissues were banned from meat and meat products regulated by many countries.

Prion diseases are characterized by accumulation of pathological prion proteins (PrP^{Sc}) [3]. Many diagnostic

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Abbreviations: AP, alkaline phosphatase; **BSE,** bovine spongiform encephalopathy; **CNS,** central nervous system; **GFAP,** glial fibrillary acid protein; **POD,** horseradish peroxidase; **NSE,** neuron-specific enolase; **PK,** protease K; **PrP^{Sc},** pathological prion; **SRM,** specific risk materials

techniques have been developed in recent years for detection of PrP^{Sc} as marker [4–6]; however, a sensitive detection method using such techniques requires sufficient PrP^{Sc} accumulation in the tissues analyzed which prevents a highly sensitive diagnosis. Therefore, proteins have to be identified for the use as indicators of CNS tissues, which can be highly sensitive detected by cheap, fast and reliable techniques.

Food controls require the highly sensitive detection of CNS tissue proteins in meat products. The detection of markers for SRM is achieved by systematic testing using ELISA [7, 8], GC-mass spectroscopic measurements [9], Western Blot analyses [10, 11], immunohistochemistry [12], real time RT-PCR approaches [13], fluorescence-based RT detection assays [14] and the immuno-PCR method [15]. These techniques are focused on the highly sensitive detection but are specific for only one CNS marker in a food product.

Since inspection is demanded by European laws, analytical assays have been introduced for the detection of indicators of CNS contamination in meat products *i.e.* the neuron-specific enolase (NSE) and the glial fibrillary acid protein (GFAP). GFAP is highly expressed in astrocyte cells [7], whereas the NSE is deposited in neurons. Both proteins are not found or are present at only very low levels in peripheral tissues making their detection appropriate for use as markers for CNS tissues for the determination of food contaminated with SRM [7]. Two different assays have been carried out using the ELISA method and the immunoblotting for routine analysis of these proteins. Thus the detection of the two antigens in one system with a capacity for multiplex analysis would be preferable. Using immunoblotting we describe the development of a novel one-step detection assay for simultaneous determination of the two indicator proteins GFAP and NSE on a single Western Blot. However, the GFAP protein and protein fragments run on gels with molecular weights similar to the NSE, whereas the GFAP bands are very close to the NSE product hindering their differentiation. By selection of appropriate primary and secondary antibodies and using the marker proteins we analyzed meat products for the presence of high-risk CNS material. The differentiation between the two antigens is made on the basis of a two-colour development process specific for the individual antigens.

2 Materials and methods

2.1 Antibodies

Indicator proteins derived from the CNS included the NSE which is detected using a monoclonal mouse anti-NSE antibody purchased as purified IgG antibody and the GFAP which is detected using a polyclonal rabbit antibody anti-GFAP (Dianova, Hamburg, Germany).

Affinity purified goat anti-mouse IgG conjugated with horseradish peroxidase (POD) and affinity purified goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) (Dianova) served as secondary antibodies.

2.2 Food and brain tissue preparations

Meat and sausages as such or with high contents of cattle meat products including minced meat, salami, goulash and calf liver sausage were acquired from local butcheries. Brain tissues of cattle which were free of prion diseases were obtained from the brain stems of six animals and were pooled to avoid individual effects. Preparations of 10% w/v homogenates occurred as described [16] with slight modifications. Briefly, tissues were weighed and homogenized in nine volumes Tris buffered saline (TBS, pH 7.4; Sigma, Germany) using pestles in glass tubes and homogenizers driven by an electric motor (VWR, Darmstadt, Germany) followed by intensive sonication (Bandelin, Berlin, Germany) for 30–60 s. Cell debris of homogenate suspensions (10% w/v) was removed by centrifugation at $900 \times g$ for 5 min and supernatants were stored at $-20^{\circ}C$ until use.

2.3 Immunoblot analysis

SDS-loading buffer was added to the samples prior to denaturation at 99°C for 5 min. Proteins were separated using 13% SDS-PAGE in a mini slab gel apparatus (Bio-Rad, Munich, Germany). After electrotransfer onto nitrocellulose membranes (Roth, Karlsruhe, Germany) using a semi-dry blotting system (Roth), non-specific binding was blocked by incubation of the membranes in TBS containing 0.05% Tween 20 (TBST) and 1% w/v non-fat dry milk powder for 1 h at room temperature. After a short washing with TBST, membranes were incubated simultaneously with antibodies, anti-NSE and anti-GFAP, in TBST containing 10% w/v LowCross powder (TBST-LC; Candor Biosciences, Weißensberg, Germany). After 12–16 h incubation the membranes were washed four times with TBST. For visualization of the immunological antigen detection enzyme conjugated secondary antibodies, POD conjugated goat anti-mouse and AP conjugated goat anti-rabbit immunoglobulin antibodies were added at appropriate dilutions and membranes were incubated in TBST-LC for 2–3 h. After washing with TBST, signals for the POD-conjugated antibody detection of NSE were visualized with diaminobenzidine as substrate according to the manufacturer's instructions. When signals were visualized, the reaction was stopped by washing the membrane five times in TBST. The binding of the AP-conjugated antibody to the GFAP-antibody complex was visualized by development of a red colour originating from Fast-Red tablets according to the manufacturer's instructions (Sigma, Steinheim, Germany).

3 Results and discussion

The detection of marker proteins which indicate contaminations of products are of considerable importance in the prevention of infections. The immunoblotting technique is frequently used for diagnostic confirmation of the presence of such markers. With this method proteins are separated by means of their molecular masses, and full length and fragmented proteins as well subtypes can be identified; however, the detection is limited to one antigen. In this study we developed an immunoblot assay for the determination of two different CNS markers, the NSE and the GFAP, on one blot. Both proteins run with similar molecular masses in immunoblots and overlay nearly with their bands but can be differentiated by a two-colour visualization (Fig. 1).

The immunoblots are incubated together with mouse and rabbit primary antibodies recognizing NSE and GFAP, respectively. This is followed by simultaneous incubation of the appropriate secondary antibodies conjugated with POD and AP, respectively, and consecutive development of the protein signals. The NSE shows up as a protein band at approximately 46 kDa [17] coloured in brown (Fig. 1A). GFAP runs in immunoblots at approximately 55 kDa, usually associated with protein bands of lower molecular masses which are believed to be proteolytic fragments and/or alternate transcripts from the single gene because GFAP may exist in different isoforms [18, 19]. The GFAP bands are red coloured (Fig. 1A). To assess the sensitivity and specificity of the two-antigen-detection procedure, proteins of meat spiked with dilutions of crude brain homogenates are separated by SDS-PAGE, immunoblotted and signals are visualized by the development of chromogenic substrates. The two proteins are clearly visualized at 0.5% of a brain homogenate and the limit of detection is at a level of approximately 0.2%. The reproducibility was demonstrated in repeated runs with various meat samples (Fig. 1A).

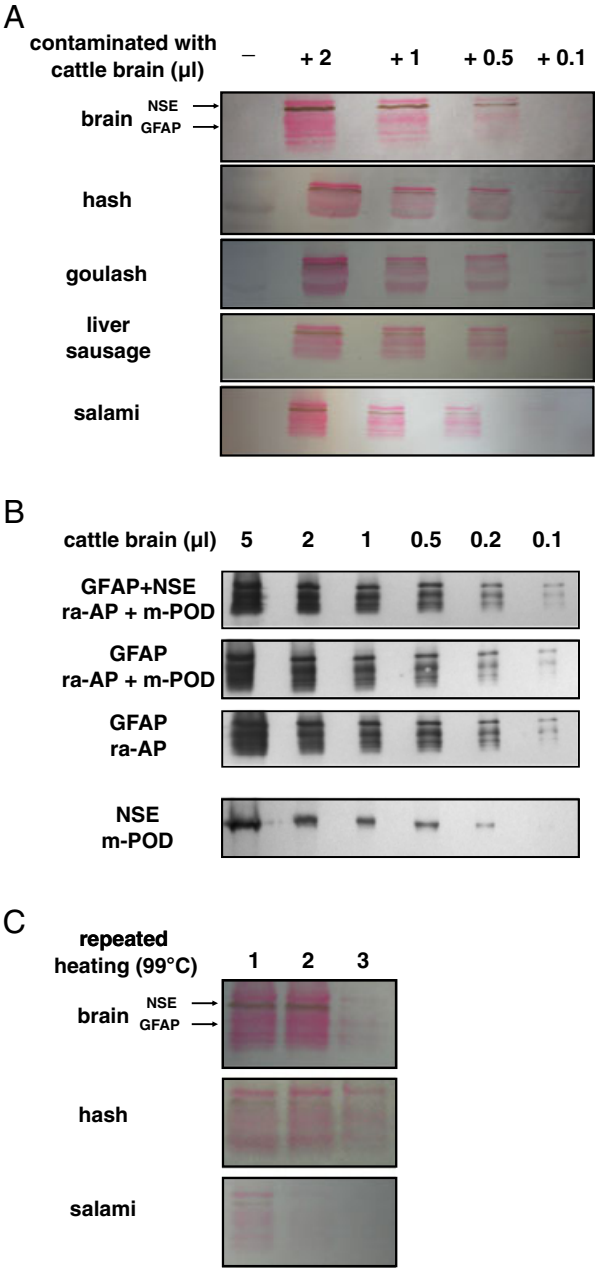

Figure 1. One-step immunoblot analysis of overlapping CNS marker proteins. (A) Homogenates of the meat products (minced meat, goulash, liver sausage and salami) (10% each) were divided into 10 µL-aliquots and proteins were loaded onto gels as pure product (–) or spiked with cattle brain homogenates (10%) in the range of 2.0–0.1 µL as indicated. Dilutions of cattle brain homogenates (10%) were used as controls (brain). Proteins were separated by electrophoresis and bands were blotted onto nitrocellulose membranes. The NSE was detected with the mouse anti-NSE antibody and the POD-conjugated secondary antibody and the signal was visualized using diaminobenzidine as substrate producing a brown colour. The GFAP was bound by the rabbit anti-GFAP antibody and the AP-conjugated secondary antibody. The immunocomplex was visualized by development of a red colour originating from Fast-Red tablets. (B) Analysing the interference of antibodies to sensitivity dilutions of a cattle brain homogenate were loaded onto gels as indicated and proteins were separated by SDS-PAGE followed by immunoblotting. For detection primary antibodies rabbit anti-GFAP (designated as GFAP) and mouse anti-NSE (NSE) were incubated single or mixed as indicated. Incubation followed with secondary antibodies anti-rabbit IgG conjugated with AP (designated as ra-AP) and anti-mouse IgG conjugated with POD, which were applied as a mix or single. GFAP signals were developed with Fast Red tablets and NSE signals came up with diaminobenzidine. (C) As model for meat processing, the stability of the indicator proteins was analyzed by repeated heating at 99°C for 5 min each. Brain homogenates as well as 10 µL-aliquots of salami and minced meat contaminated with 2 µL brain homogenates (10%), respectively, were heated once (1), twice (2) and three times (3) for 5 min each followed by cooling to 4°C for at least 1 h. Brown proteins are the NSE signals and the red bands are the GFAP signals.

The method allows semi-quantification of the results. Defined brain tissue homogenates of one batch were used as standards. In dilutions signals of NSE and GFAP were detected in repeated gel runs and separated signal visualizations. The spiking of meat samples with known amount of a brain homogenate enabled an assessment if the test accurately identified the presence of CNS tissues. It is of great importance to evaluate the specificity of the test so that no signals come up when no CNS material is present. Background signals were analyzed using meat samples without spiking with brain homogenate. Very faint bands were observed under these conditions, however, these showed clearly deviant molecular masses than the NSE and GFAP bands.

To analyze the interference of the antibodies that are used coexistent in the assay the signals of dilutions with antibody combinations were detected. In the one-step detection assay no difference is observed in the limits of detection when blots are incubated with single or both antibodies as well secondary antibodies followed by signal development (Fig. 1B). The antigen determination is specific without showing any cross-reactivity between the antibodies or against other proteins and no background signals came up. The colour development is specific for each chromogenic substrate and no background and false positive signals are detected (not shown). GFAP was determined in dilutions of 0.1 μL of a 10% brain homogenate, whereas the NSE detection limit was at the range of 0.2 μL in the same sample. A sensitive detection is influenced not only by the affinity of antibody to antigen but also by the expression rate of the antigen itself when using crude tissue homogenates.

The recovery of proteins is often limited by sample storage. However, there is no signal loss for NSE and GFAP when protein samples are stored at −20°C for 1–2 wk or for at least 3 days at 4°C (not shown) thus verifying the stability properties described previously [20]. On the other hand, repeated heating produces a marked denaturation of the proteins and signal intensities decreases appreciably. The signal reduction depends on the meat product used for spiking (Fig. 1C). Heating of minced meat contaminated with brain tissues results in high signal reduction with both proteins GFAP and NSE. Protein signals in salami are dramatically reduced even after the first heating. These data indicate that the ability to detect proteins will fall when raw meat is prepared during meat processing and determination of SMR. Interestingly, GFAP seems to be slightly more stable than NSE.

Several assays have been described for the detection of NSE and GFAP as indicators of CNS tissues in food products. Commercial kits are available for determination of each of the antigens. Applying these methods, CNS proteins can be detected in a range of concentrations of 0.1–0.5% [21]. The limitations of the techniques are in a similar range than the one-step immunoblot approach. This discrepancy of the detection limits analyzed by the different test systems are possibly be due to preparations of the meat and the pre-treatments as cooking and applications of ingredients.

The Ridascreen Risk Material kit (r-biopharm, Germany) is an ELISA that detects the presence of GFAP. The method based on a sandwich ELISA technique offers the facility to load high protein amounts on the matrix. Specific antigens are bound to pre-coated antibodies and disruptive and unbound proteins will be released by washing steps. Values of the results are given in numbers which allow precise interpretation of specific data. However, this technique is not usable for simultaneous detection of two different antigens.

The Brainostic (ScheBo, Germany) test is an immunoblot test to detect NSE. The separation of the proteins by SDS-PAGE allows the differentiation of proteins with various molecular masses and the specific detection of the antigen by immunoblotting. The procedure is more labour-intensive than the ELISA technique and the interpretation of results is rather subjective above all when comparing bands with differing intensities. But all for that the technique is highly consistent in repeated gel runs. Furthermore, the immunoblot method is highly flexible in such a way as to enable the detection of different antigens on one immunoblot without loss of specifity. False positive and background signals which possibly may come up by unspecific antibody bindings can be selected easily, because protein bands mostly have aberrant molecular masses than the antigens to be analyzed. In contrast, such signals detected in an ELISA system result in high data values which cannot be interpreted and analyzed.

The novel multiplex immunoblot detection offers many experimental and analytical benefits. With conventional techniques the simultaneous detection of two proteins with similar molecular masses requires either separated blots or blots that will be stripped and re-probed. Separated blots need additional and accurate gel loading as well as an efficient and reproducible protein-transfer onto the membrane. However, this cannot always be guaranteed in the case of low amounts or complexes under certain conditions. Stripping of antibodies bound to the antigen can cause varying loss and denaturation of proteins resulting in a reduction in signal intensity and a decrease in sensitivity, whereas insufficient stripping results in non-specific and non-identifiable bands [22]. The novel one-step protein detection assay of several antigens requires two chromogenic substrates developing different colours which arise from the specific antigen signals on immunoblots. This conventional detection assay for the determination of two antigens involves the direct determination of the antigens on the immunoblots and requires only one-step incubation of mixed primary and mixed secondary antibodies. Under certain pre-treatment conditions proteins are denatured and antigenicity is decreased or will be lost. In raw or only moderately heated sausages GFAP and NSE were detected at a limit of 0.5% CNS tissue [23].

For strongly heated meat the limit decreased to 2% for NSE but remained constant for GFAP determined with single commercial test kits [23]. In this context the developed multiplex one-step detection assay described here offers the advantage to detect several antigens of which at least one will be stable and resist to denaturation conditions even when other indicators are denatured under these conditions.

Food controls require a highly sensitive detection of CNS tissues in meat products as marker proteins for SRM, which has to be removed during slaughtering processes in order to prevent transmission of BSE to humans. Indicators should be detected reliably by cheap, fast and sensitive methods. PrP^{Sc} are the infectious agents for the disease [3] and therefore an effective marker for diagnosis. However, a sensitive detection method requires sufficient PrP^{Sc} accumulation in the tissues analyzed and that the prion has a high resistance to protease K (PK) treatment to differentiate between physiological prions and PrP^{Sc}. Though prion field isolates and strains vary considerably in their stability to PK [24], and PK sensitive and infectious PrP^{Sc} proteins have been identified [25] which prevent a definite diagnosis. Thus PrP^{Sc} is not a reliable marker for highly sensitive detection.

The multiplex assay with detection of two antigens on one immunoblot demonstrates simplicity and rapidity, and specialized and expensive imaging systems are not needed. Thus the immunoblotting approach is applicable for routine diagnosis with sensitive qualitative detection. After standardization the technique can also be used for highly sensitive detection of other marker proteins which indicate the very early stage of human and animal diseases or contaminations of food and medical products.

4 Concluding remarks

The novel multiplex immunoassay enables determination of two CNS marker proteins, the NSE and the GFAP, in a one-step process on a single immunoblot. The proteins were detected by immunological antibody binding and visualized by reactions with chromogenic substrates. Specific signals in two different colours enabled the unambiguous differentiation of the proteins. The two-colour assay can be used for the simultaneous detection of proteins when the molecular masses are very similar. Multiplex assays for detection of disease marker proteins are of utmost importance for food safety and consumer protection against transmissions and infections and have considerable potential for application in the food industry, veterinary facilities and in human health.

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5 References

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