

## An Enzyme-Linked Immunosorbent Assay for Glial Fibrillary Acidic Protein as an Indicator of the Presence of Brain or Spinal Cord in Meat

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### ABSTRACT

The current methods to detect central nervous system (CNS) tissue in blood, lungs, or meat are cumbersome, time consuming, and costly. The objective of this study was to use glial fibrillary acidic protein (GFAP), which is restricted to the CNS, in an enzyme-linked immunosorbent assay (ELISA) for the detection of CNS tissue in blood and muscle from beef cattle. Bovine brain, cerebral cortex, spinal cord, sciatic nerve, diaphragm, blood clots, and other skeletal muscle were obtained from three animals at slaughter. The limit for detection of GFAP was approximately 1.0 ng and the standard curve was linear up to 40 ng. Tissue samples gave responses parallel to the GFAP standard, suggesting that standard and unknown samples were immunoreactively identical. No GFAP was detected in skeletal muscle (ground beef, shoulder clod, and diaphragm) and blood clots. Trace amounts (13.5 to 51 ng/mg) were present in sciatic nerve. In contrast, high levels of GFAP (55 to 220 µg/mg) were present in spinal cord, cerebral cortex (17 µg/mg), and whole brain (9 to 55 µg/mg). In a storage study using two animals in two separate studies, immunoreactive GFAP was detectable for up to 8 days at 4°C in all tissues containing neural elements. Thus, mixtures of muscle with spinal cord or brain retained almost 80% of their immunoreactivity after 8 days at 4°C, while brain and spinal cord alone retained approximately 50% and 25%, respectively, of their initial activities. In a repeat experiment, 80 to 100% of the initial activity was retained in these tissues after 8 days at 4°C. The results of the current study demonstrate that the GFAP ELISA provides a valid and repeatable method to detect CNS tissue contamination in meat.

The presence of brain or spinal cord as an inadvertent contaminant of meat may result from the stunning of live-stock or the preparation of advanced meat recovery meat from vertebral column (1, 5, 6, 10). If the bovine spongiform encephalopathy (BSE) agent was present in the animals being processed, this could be a food safety concern. Even if the BSE agent was not present, consumers prefer that regulatory agencies monitor the presence of central nervous system (CNS) tissue in beef (5). The current methods to detect CNS tissue in blood, lungs, or meat include gross tissue dissection and visual examination (1, 6), analysis of cholesterol (7), or histological preparation and examination of miniscule samples (5). These methods are cumbersome, insensitive, time consuming, and costly, which prevent the examination of many samples to evaluate cattle-stunning methods or preparation techniques for AMR.

Glial fibrillary acidic protein (GFAP) is the major protein constituent of glial filaments in differentiated astrocytes, which are restricted to the CNS (4). The antigenicity of GFAP has permitted the preparation of highly avid and specific polyclonal and monoclonal antibodies (4). GFAP immunohistochemistry has been used for the diagnosis of astrocytic tumors, the study of astrocyte development, the

study of astrocytic gliosis, and the study of CNS regeneration and transplantation (3).

Detailed protocols have been reported for quantifying mouse GFAP by a microtiter plate-based sandwich enzyme-linked immunosorbent assay (ELISA) (8). The objective of this study was to examine the efficacy of the GFAP ELISA for the detection of CNS tissue in blood and muscle from beef cattle. The sandwich ELISA is a simple, cost-effective, safe, and efficient method to determine minute quantities of CNS tissue in non-neural tissues. We report here the application of this specific, sensitive technique to the detection of neural tissue contamination of beef.

### MATERIALS AND METHODS

**Tissue collection.** The presence of GFAP in a variety of bovine tissues was examined in tissues collected from three animals. Brain, spinal cord, sciatic nerve, diaphragm, blood clots, and skeletal muscle were obtained at slaughter at the Colorado State University Animal Sciences abattoir. Tissues were either quick-frozen in liquid nitrogen and stored at -70°C or were kept on ice for transport to the laboratory for processing and analysis. Whole brain was cleaned of membranes and blood clots, rinsed in 0.9% NaCl, and then processed by blending for 20 s. in a Robot Coupe R172 (Robot Coupe USA, Inc., Ridgeland, Miss.) food processor. Diaphragm was processed in an Osterizer 10-speed blender (Sunbeam-Oster Products, Wood Ridge, N.J.) in a one-cup glass jar for approximately 1 min. Spinal cord and sciatic nerve were cut thoroughly with scissors until a fine mince was obtained. These

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minces were solubilized by homogenizing in 10 volumes of 1% sodium dodecyl sulfate (SDS) at 85 to 90°C using two 10-s bursts with a Virtishear homogenizer (Virtis Co., Inc., Gardiner, N.Y.).

**Stability of GFAP.** To examine the stability and detection of GFAP in tissues stored at 4°C, neural tissue alone or a mixture of neural and muscle tissue was prepared and sampled at intervals up to 16 days. The storage study was performed twice using two separate preparations of tissues from two animals. Mixtures of spinal cord or brain and muscle were prepared by adding 1 g of minced neural tissue to 99 g of ground beef and processing with the Oster blender, as above, to form a homogenous paste. After processing, mixtures of neural tissue and muscle, as well as neural and muscle tissue alone, were placed in plastic storage bags and stored at 4°C. Two 0.5-g samples from each tissue were removed after 0, 2, 4, 8, and 16 days at 4°C. Identical aliquots of muscle were collected after 0, 8, and 16 days at 4°C. Aliquots were frozen at -20°C until they were thawed, homogenized in hot SDS, and analyzed in a single ELISA. Protein analysis of the samples was performed with the Bradford dye reagent (Bio-Rad, Hercules, Calif.) using bovine serum albumin as the standard. Although 1% SDS will interfere in this protein assay, dilution of samples at least 10-fold prior to analysis reduces SDS interference to a minimum (2).

**ELISA reagents.** The following reagents were used in the current study. Reagent A was phosphate-buffered saline (PBS): One packet of PBS (Pierce Chemical Co., Rockford, Ill.) was dissolved in 500 ml of deionized water (final concentration = 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4). Reagent B consisted of PBS + 0.5% Triton X-100 (Bio-Rad). Reagent C was PBS + 5% Carnation nonfat dry milk (local grocery store). Reagent D was PBS + 5% powdered milk + 0.5% Triton X-100. Reagent E was 1:400 polyclonal anti-GFAP in PBS (Dako, Corporation, Carpinteria, Calif.). Reagent F was 1:500 monoclonal anti-porcine GFAP (Boehringer Mannheim, Indianapolis, Ind.) prepared in reagent D. Reagent G was alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.) in reagent D (1:3,000 dilution). Reagent H was *p*-nitrophenylphosphate substrate (Bio-Rad) prepared according to manufacturer's instructions.

**Preparation of standards and samples for ELISA.** Bovine GFAP standard (American Research Products, Boston, Mass.) was dissolved in distilled water and serial dilutions of the standards were prepared in reagent B. The final concentrations of each standard were 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 ng/100  $\mu$ l. For each ELISA plate (Immulon-2, Dynex Technologies, Chantilly, Va.), reagent B (blank) and each of the nine standards, in duplicate, were added to the first 20 wells of the plate. Diluted standards could be stored at 4°C (not frozen) for up to 10 days.

Samples were prepared as described above by homogenizing in 10 volumes of 1% hot SDS. Samples were diluted in reagent B for protein analysis and ELISA testing. Muscle samples were diluted at least 1:6 (~1 mg protein/ml) to avoid interference of SDS in the ELISA, while neural tissue was diluted at least 1:3,000 (~1  $\mu$ g protein/ml). Two 10-fold serial dilutions of the minimal dilutions above provided three samples that were within the range of ELISA sensitivity. Samples (100  $\mu$ l/well) were analyzed in duplicate at each dilution.

**Assay procedure.** Reagent E (100  $\mu$ l) was added to each microplate well and the plate was incubated at 37°C for 1 h. (This step could be done at the beginning of the assay or the day before and the plate refrigerated overnight at 4°C.) After incubation, the

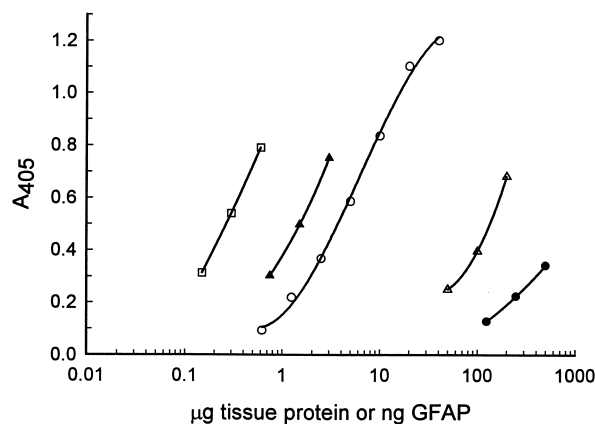


FIGURE 1. Standard curve for the GFAP ELISA and parallel dose-response curves for neural tissue alone and in combination with ground beef. Spinal cord ( $\square$ - $\square$ ), brain ( $\blacktriangle$ - $\blacktriangle$ ), or mixtures of ground beef and spinal cord ( $\triangle$ - $\triangle$ ), or ground beef and brain ( $\bullet$ - $\bullet$ ) were compared at different dilutions with GFAP standard ( $\circ$ - $\circ$ ). Neural tissue and ground beef were mixed at a ratio of 1:100 before ELISA analysis. Results are from a representative ELISA of a single individual.

plate was emptied and blotted on absorbent paper to remove excess fluid. Plates were washed four times with 200  $\mu$ l per well reagent A and blotted after each wash. Reagent C (100  $\mu$ l) was added and the plate incubated for 1 h at room temperature; then the plate was emptied, blotted, and standards and samples were added in a volume of 100  $\mu$ l per well and incubated for 1 h at room temperature. The plates were washed four times with 200  $\mu$ l/well reagent B before the addition of 100  $\mu$ l/well reagent F and incubated for 1 h at room temperature. The plates were washed four times with 200  $\mu$ l/well reagent B and then incubated for 30 min at room temperature with 100  $\mu$ l/well reagent G. After four washes with 200  $\mu$ l/well reagent B, plates were incubated for 20 min at room temperature with reagent H (100  $\mu$ l/well). The reaction was stopped by the addition of 100  $\mu$ l/well 0.4 N NaOH and absorbance at 405 nm was recorded using an Elx 800 plate reader (Biotek Instruments, Inc., Winooski, Vt.).

## RESULTS AND DISCUSSION

Recent occurrences of BSE have heightened awareness of neural diseases that can be transmitted by consumption of neural tissue (5). At present, neural tissue in meat products is assessed by gross visual or histological examination. Both methods are subjective and histological examination is expensive, time consuming, and subject to false negatives due to the small area that can be effectively examined. We report here the use of an objective, easily performed immunoassay for the presence of CNS tissue in meat products.

A typical standard curve for the GFAP ELISA generated during these studies is shown in Figure 1. The limit for detection of GFAP (absorbance  $\geq 0.05$ ) was approximately 1.0 ng and the curve was linear up to 40 ng. Correlation coefficient ( $r^2$ ) values of 0.994 to 0.999 were obtained with a cubic fit (12 assays). Intra-assay variation values ranged from 3.25 to 4%. Figure 1 also shows that the samples prepared for analysis gave responses parallel to the GFAP standard, suggesting that standard and tissue samples were immunoreactively identical.

Dose-response curves for the three neural tissues test-

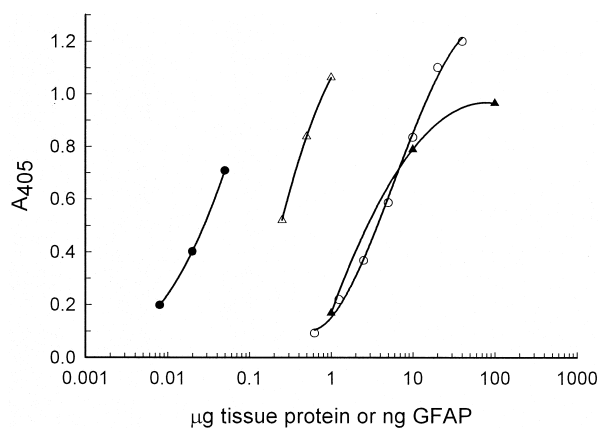


FIGURE 2. Comparison of CNS and peripheral nerve with GFAP standard. Dose-response curves were generated with spinal cord (●—●), brain (△—△), and sciatic nerve (▲—▲) for comparison to GFAP standard (○—○). Results are from a representative ELISA of a single animal.

ed compared to the GFAP standard are shown in Figure 2. Tissues of the CNS elicited parallel dose-responses at 0.008 to 0.05 (spinal cord) and 0.2 to 0.8 (brain)  $\mu\text{g}$  protein. In contrast, the sciatic nerve gave a nonparallel dose-response at very high concentrations of tissue protein (1 to 100  $\mu\text{g}$ ). This indicates that very high levels of pure peripheral nerve may cross react in the ELISA assay. The nonparallelism of sciatic tissue to the GFAP standards and to CNS tissue may be due to cross reaction with a related antigen or from interference by non-GFAP sciatic nerve components. We do not feel that this presents a realistic problem in the analysis of CNS contamination of meat products. Sciatic nerve (and presumably other peripheral nerves) would be detected in this assay only if present as the primary component of the meat sample. Thus, if sciatic nerve comprised 50% of the meat sample, a false-positive value of  $\leq 25$  ng/mg GFAP would result. This is about one-fourth of the value resulting from a 1% contamination of meat with CNS tissue. Visual inspection of meat product used for sampling, careful sampling technique, and the use of a cutoff value for significant

GFAP contamination of about 50 ng/mg will circumvent this problem.

Table 1 presents the results of GFAP quantitation in different bovine tissues. The range in values presented represent the results from two separate ELISAs on two to three individuals. The large variation in GFAP levels are likely due to individual differences and the amplifying effects of large dilutions of the brain and spinal cord samples. No GFAP was detected in tissues peripheral to the CNS including skeletal muscle (ground beef, shoulder clod, and diaphragm) and blood clots. Very low levels of GFAP were detected in sciatic nerve samples. Interestingly, neck muscle samples exhibited low GFAP levels that probably are the result of contamination from spinal cord during the slaughter process. In contrast, high levels of GFAP were present in spinal cord, cerebral cortex, and whole brain. These results confirm that GFAP is restricted to the CNS in cattle and is not present in the non-neural tissues sampled. Thus, GFAP represents an excellent molecular marker for CNS tissue, as it is restricted to the CNS where it is present in high concentrations.

To examine further the utility of the GFAP ELISA in an industry setting, samples of ground beef, brain, and spinal cord were collected at slaughter and stored alone or as mixtures at 4°C for up to 16 days. As shown in Figure 3, immunoreactive GFAP was readily detectable for up to 8 days of storage at 4°C in all tissues containing neural elements. Mixtures of muscle with spinal cord or brain retained almost 80% of their immunoreactivity after 8 days at 4°C, while brain and spinal cord alone retained approximately 50% and 25%, respectively, of their initial activities. In a repeat experiment, using tissues from another animal, 80 to 100% of the initial activity was retained in these tissues after 8 days at 4°C. It is presumed that immunoreactivity of the stored samples declines due to proteolytic activity or oxidation of the samples. Ground beef alone had no GFAP immunoreactivity in either experiment.

The results of the current study demonstrate that the GFAP ELISA provides a valid and repeatable method to detect CNS tissue contamination in meat. The assay is ex-

TABLE 1. Estimates of GFAP immunoreactivity in bovine tissues<sup>a</sup>

Tissue	Assay dilution	GFAP (ng/mg)
Spinal cord	1:300,000	55,000–220,000
Brain	1:30,000	9,000–55,000
Cerebral cortex	1:30,000	17,000
Sciatic nerve <sup>b</sup>	1:250	13.5–51
Diaphragm	1:100	0
Shoulder clod	1:100	0
Neck muscle	1:100	2.8
Ground beef	1:100	0
Ground beef plus spinal cord (100:1)	1:100	90–130
Ground beef plus brain (100:1)	1:100	80–200
Blood clot	1:100	0

<sup>a</sup> Concentrations of GFAP were measured in tissues of two to three animals by sandwich ELISA at the dilutions noted. The range of values is presented on a wet tissue weight basis.

<sup>b</sup> Nonparallel to standard curve.

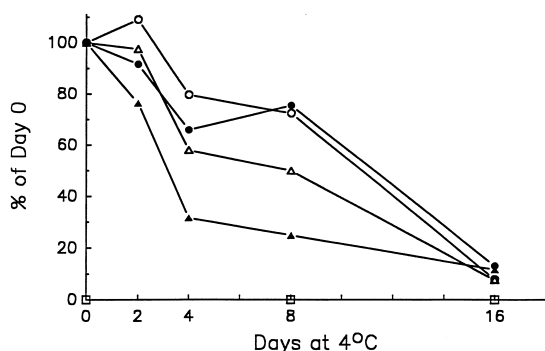


FIGURE 3. Effects of storage at 4°C on GFAP immunoreactivity in neural tissue alone or when mixed with ground beef. Data are presented as the percentage of day 0 (day of slaughter) GFAP immunoreactivity at the indicated day of storage. Day 0 absorbance values at 405 nm were approximately 0.4 to 0.8. Results presented represent values derived from a single experiment using tissues from one animal. All data points are the mean of duplicate samples that varied less than 5% from the mean. Spinal cord (▲-▲), brain (△-△), ground beef (□-□), and 1:100 mixtures of spinal cord with ground beef (●-●), and brain with ground beef (○-○) are shown.

tremely sensitive and is specific for CNS tissue. No cross reaction with skeletal muscle or blood clots was detected, and GFAP levels in peripheral nerves (sciatic) were minimal. Thus, GFAP concentrations in sciatic nerve were 0.06 to 0.5% of those in spinal cord and brain and 100- to 1,000-fold more sciatic tissue was required to provide a response equivalent to brain and spinal cord in the ELISA (see Table 1). Tissues containing GFAP can be stored at refrigerator temperature for up to 8 days postmortem and still be used for analysis. An improved fluorescent version of the GFAP ELISA has been developed that is 20 to 30 times more sensitive than the assay used for the current study (O'Callaghan, unpublished data). This would allow the detection of subnanogram amounts of GFAP in tissues of interest. Although the quantitative aspects of the GFAP ELISA were emphasized in the current validation study, application in an industry setting could be much simpler. Thus, two to three standards and two dilutions of tissue samples would provide a yes or no evaluation of the pres-

ence of neural tissue in meat products. Adaptation of an automated robotic system for the GFAP ELISA, which would simplify analysis of multiple samples, has been reported (9). The use of this system would allow the rapid screening of thousands of samples in a simple repeatable assay. Indeed, the simple visual observation of color in the ELISA would be sufficient to establish the presence of GFAP (and thus CNS tissue) in meat products.

In conclusion, a simple, safe, sensitive, and specific assay for the detection of CNS tissue in meat products has been developed. It is repeatable, can be used in tissues stored at refrigerator temperature for up to 8 days, and is readily amenable to automation and analysis of hundreds of meat samples daily.

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