Optimization of Sample Preparation and Storage Conditions for a Biochemical Assay to Detect Neural Tissue in Meat

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SUMMARY

Studies were undertaken to examine sample preparation and storage conditions for a glial fibrillary acidic protein (GFAP) immunoassay (ELISA) for detection of neural tissues. Altering the homogenization conditions by buffering 1% SDS to pH 7.4 and decreasing the temperature from 95°C to 50°C allowed for maximum GFAP detection in the ELISA. Homogenization at 50°C in 1% SDS in PBS, pH 7.4 maximized GFAP concentration for both freeze-dried and frozen neural samples. In general, freeze-drying interferes with the detection of GFAP in the ELISA. Even under optimum homogenization conditions, the GFAP concentration of freeze-dried spinal cord was only about half that of frozen spinal cord. The storage of standards at 4°C for 48 hr reduces the amount of GFAP detected by the F-ELISA. Therefore, standards can be stored as either 320 stock or working stock and used for two days before new standards need to be made. The storage of homogenates at -20°C results in a large decrease in GFAP concentration after only a few days. Centrifugation of homogenates increased GFAP concentration and stability when samples were frozen and thawed.

Key words: BSE, Immunoassay, GFAP, Meat, Neural Tissue

INTRODUCTION

The presence of brain or spinal cord as an inadvertent contaminant of meat may result from the stunning of livestock, the splitting of the carcass or the preparation of advanced meat recovery (AMR) products from the vertebral column. In light of current consumer concern about bovine spongiform encephalitis (BSE), a disease that may be transmitted by consumption of CNS tissue, a reliable analytical test for CNS tissue in meat products is essential to ensure consumer confidence and allay consumer fears of BSE in meat products. A method to detect the presence of CNS tissue in meat products has been developed in our laboratory (Schmidt et al., 2001). The method uses an enzyme-linked immunosorbent assay (ELISA) for the detection of glial fibrillary acidic protein (GFAP), an antigen which is highly, but not completely, restricted to astrocytes in the CNS. This assay provides a method to detect small amounts of CNS tissue in meat products that is simple, cost effective and efficient. The assay is capable of detecting sub nanogram levels of GFAP and can be used on product samples that have been stored at 4°C for up to 8 days. The current study was designed to optimize the conditions used for neural tissue preparation and storage in the fluorescent ELISA.

METHODS

Bovine brain and spinal cord were freeze-dried to test the method as a means of sample storage. To make the freeze-dried brain 4 bovine brains were homogenized for 2 minutes in a Robot Coupe R172 (Robot Coupe USA, Inc., Ridgeland, Miss.) food processor. The processed brain (2.2 lbs) was placed in a plastic container and freeze-dried in a Labconco Freezone 12. To make freeze-dried spinal cord the same procedure was followed except more than 10 bovine spinal cords were used. For comparison a spinal cord pool consisting of 7 g tissue from each of 10 spinal cords was prepared by homogenization in a blender until a uniform mixture was formed. This preparation was stored frozen at -20°C.

Freeze-dried bovine brain and spinal cord samples were prepared with a variety of homogenization conditions to determine which resulted in the highest detectable GFAP concentration. Freeze-dried spinal cord preparations were then compared with those from frozen bovine spinal cord. The freeze-dried brain and spinal cord were diluted 1:10 in either 1% sodium dodecyl sulfate (SDS, Sigma Chemical Co., St. Louis MO), 1% Triton X-100 (Bio-Rad) or 1% Tween 20 (Fisher Scientific, Fair Lawn NJ) and homogenized at 50°C or 95°C. Additional freeze dried brain and spinal cord samples were diluted 1:10 in phosphate-buffered saline (PBS, Pierce Chemical Co., Rockford, IL) and shaken at room temperature for 3 hours. One ml of the freeze-dried brain or spinal cord suspended in PBS was homogenized in 9 ml of either 1% SDS, 1% Triton X-100 or 1% Tween 20 at 50°C or 95°C. Each of these samples was analyzed in the fluorescent ELISA, exactly as detailed in Schmidt et al (2001), with the freeze-dried brain samples at a final dilution of approximately 1:50,000 and the freeze-dried spinal cord samples at a final dilution of approximately 1:150,000. In order to calculate GFAP concentration in ng/mg wet weight, the dry weight result was multiplied by 0.18 for brain samples and 0.35 for spinal cord samples to compensate for the loss of water weight during lyophilization.

Freeze-dried and frozen bovine spinal cord samples were homogenized under different conditions to maximize GFAP detection. For each sample 1 g of freeze-dried or frozen spinal cord was homogenized in 10 ml of either 1% SDS, 0.5% SDS, 1% SDS in PBS or 0.5% SDS in PBS at either room temperature (22°C), 37°C, 50°C or 95°C. Each of these samples was then analyzed in the F-ELISA diluted at 1:100,000, 200,000 and 400,000. For comparison, the freeze-dried spinal cord prepared above by suspension in PBS and then homogenizing in 1% SDS was analyzed in the F-ELISA diluted at 1:500,000, 750,000 and 1,000,000. To examine storage stability at 4°C, GFAP standard at 3.2 µg/ml (320 stock) or at 0.75-9.6 µg/ml (working stock) were assessed in the F-ELISA after storage for up to one week. All standards were diluted with PBS containing 0.5% Triton X-100.

To examine storage stability of sample homogenates at -20°C, two 1 g

samples of frozen or freeze-dried spinal cord were homogenized in 10 ml 1% SDS in PBS at 50°C. One homogenate was aliquoted into 100 µl aliquots and the other was centrifuged for 20 min at 7500 r.p.m. (10,400 x g) at 4°C. The supernatant was aspirated, divided into 100 µl aliquots and stored at -20°C. An aliquot from each of the batches of spinal cord was run on the F-ELISA once a week for 6 weeks. The effect of freeze-thawing the homogenates 1, 2, 5 and 10 times was also examined.

RESULTS

The results of homogenizing freeze-dried brain and spinal cord in 1% SDS, 1% Triton X-100 or 1% Tween 20 show that in all cases homogenization in SDS resulted in a higher amount of GFAP detected (Table 1). The study also showed that more GFAP was detected when homogenized at 50°C as opposed to 95°C. When the freeze-dried material was suspended in PBS prior to homogenization in 1% SDS, the amount of GFAP detected increased even further. Except for spinal cord at 50°C, suspension in PBS prior to homogenization in 1% SDS at least doubled the amount of GFAP detected. We then compared the effects of homogenization temperature and pH on GFAP detection in frozen and freeze-dried neural tissues. As shown in Table 2, in both the freeze-dried and frozen spinal cord the maximum values of GFAP were detected when homogenized in 1% SDS in PBS at 50°C. Even under optimal conditions the detectable GFAP concentration in freeze-dried spinal cord was only about half that of frozen spinal cord.

As shown in Figures 1 and 2, when GFAP was stored at 4°C as a 320 stock solution or in working dilutions, the relative fluorescent units (RFU) for each value decreased after the second day of storage. The RFU values for three points in the mid-range of the standard curve decreased after storage longer than two days.

The effect of centrifuging freeze-dried and frozen spinal cord homogenates and storing them for up to 39 days at -20°C was also examined. After one week of storage only 49.3-63.8% or activity remained (Figure 3). After 39 days, the homogenates lost 47-76% of their GFAP activity. In general, the centrifuged homogenate had a higher level of GFAP then the non-centrifuged for both freeze-dried and frozen spinal cord. On average the GFAP concentration of the non-centrifuged samples was 89% and 76.3% of the centrifuged spinal cord for freeze-dried and frozen samples respectively. The effect of freezing and thawing the homogenates was to reduce GFAP concentration. This was more apparent in freeze-dried samples than in frozen and for the non-centrifuged than the centrifuged samples. Centrifuging the homogenates resulted in a GFAP concentration that was stable for up to five freeze thaw cycles.

DISCUSSION

The results of the current optimization studies demonstrate that the pH and temperature of homogenization are critical for maximum detection of GFAP in the F-ELISA. This is particularly true for neural tissues that have been freeze-dried to provide a stable biological source of GFAP. It is essential that neural tissue prepared as a lyophilized powder is suspended in phosphate buffered saline at pH 7.4 and homogenized at 50°C for optimal detection of GFAP. Homogenization of frozen (or fresh) neural tissues at 50°C in 1% SDS buffered to pH 7.4 provides results equivalent to those seen with unbuffered SDS at 95°C. Use of lower homogenization temperatures provides a technique which avoids the dangers to personnel of dealing with hot solutions and provides milder, safer sample preparation conditions.

We also examined the effects of storage of GFAP standards on the F-ELISA. These studies showed that storage of GFAP standards at 4°C for longer than 2 days resulted in a reduction in fluorescence in the assay and a right-ward shift in the GFAP standard curves generated with aged standards. We concluded that GFAP standards must be prepared and used within two days of dilution in order to maintain the repeatability and sensitivity of the F-ELISA.

Neural tissue homogenates prepared in hot 1% SDS are a heterogeneous mixture of soluble and insoluble material. The GFAP antigen is localized in the cell membranes of glial cells, which must be solubilized in detergent before assay. Thus, we examined the effects of centrifuging the homogenates to remove insoluble cell debris. Neural tissue extracts prepared by centrifugation had increased GFAP concentrations and were more stable when subjected to freeze-thaw cycles. This suggests that crude homogenates contain substances that interfere in the ELISA, resulting in lower apparent GFAP concentrations. Conversely, when non-centrifuged crude homogenates are subjected to several freeze-thaw cycles, the presence of intact tissue may be a source of proteolytic enzymes or inhibitory substances that are released during the freeze-thaw cycles and reduce the amount of detectable GFAP. Centrifugation removes the intact tissue and enhances stability during freeze-thaw cycles.

In sum, the current studies demonstrate that sample preparation and storage are crucial components of a sensitive and repeatable assay for neural tissue. Examination of the homogenization pH and temperature, as well as storage stability of neural tissues and GFAP standards have revealed optimum conditions which enhance the performance of the GFAP fluorescent ELISA.

LITERATURE CITED

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Table 1. Effects of detergent, temperature of homogenization and pH on GFAP detection in freeze-dried neural tissue in the F-ELISA. Freeze-dried bovine brain and spinal cord homogenized in three different detergent solutions at two different temperatures. Samples were either suspended in PBS prior to being homogenized (PS) or not (NS). Values shown are the average with the percentage CV and number of duplicate measurements in parentheses.

Tissue	°C	Treatment	GFAP (ng/mg wet weight)		
			1% SDS	**1% Triton**	**1% Tween**
Brain	50	NS	349.1 (8.7; 2)	330.0 (6.4; 3)	224.2 (34.7; 2)
		PS	801.8 (6.5; 2)	181.7 (4.6; 4)	231.6 (7.9; 3)
	95	NS	137.5 (6.8; 3)	37.5 (28.4; 5)	37.9 (42.5; 4)
		PS	827.5 (16.9; 2)	159.7 (8.1; 3)	194.1 (9.4; 4)
Spinal Cord	50	NS	5189.5 (*; 1)	695.9 (6.4; 3)	1068.5 (10.7; 3)
		PS	7099.7 (1.1; 2)	143.8 (8.7; 3)	191.2 (4.0; 4)
	95	NS	1457.2 (4.8; 2)	41.0 (10.6; 3)	35.9 (49.5; 3)
		PS	6637.7 (2.6; 2)	139.3 (10.7; 4)	223.3 (6.8; 4)

* indicates that only one value was within the linear range of the standard curve, hence there is no variation

Table 2. Effect of homogenization temperature and pH on detection of GFAP in freeze-dried (FD) and frozen (FZ) spinal cord. Freeze-dried bovine spinal cord was homogenized in three different detergent solutions at four different temperatures. The detergent solutions were 1% SDS, 1% SDS diluted in PBS and suspending the spinal cord in PBS prior to homogenizing in 1% SDS. Frozen bovine spinal cord was homogenized in either 1% SDS and 1% SDS diluted in PBS at four different temperatures. Values shown are the average with the CV in parentheses.

Temperature (°C)	Sample	GFAP (ng/mg wet weight)		
		1% SDS	**1% SDS/PBS**	**PBS then SDS**
22	FD	988.9 (20.7%)	960.1 (6.6%)	——
	FZ	5056.7 (15.1%)	7284.0 (2.5%)	——
37	FD	883.9 (24.2%)	2056.1 (6.7%)	——
	FZ	3675.0 (8.3%)	6390.0 (0.7%)	——
50	FD	977.6 (20.1%)	3508.4 (2.5%)	2143.8 (5.4%)
	FZ	4654.5 (15.9%)	6729.0 (7.0%)	——
95	FD	464.1 (18.9%)	540.4 (6.6%)	2784.3 (5.2%)
	FZ	5088.0 (12.2%)	5178.7 (9.8%)	——

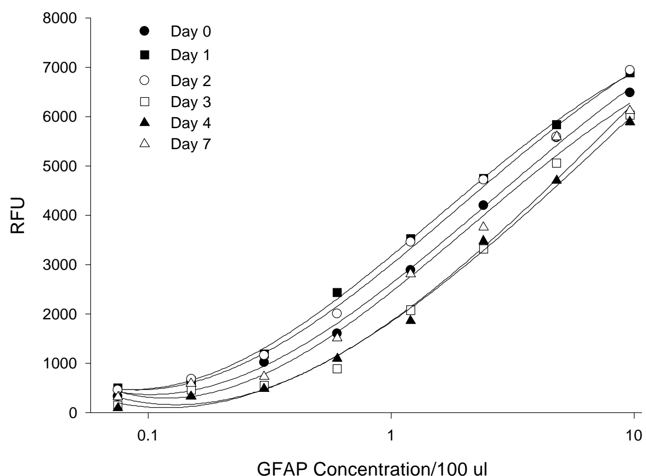

Figure 1. GFAP Standard was stored in the following dilutions, 9.6, 4.8, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075 ng/100 ul for a varying amount of time at 4°C. GFAP concentration starts to decline after storage for more than two days. RFU = Relative fluorescence units

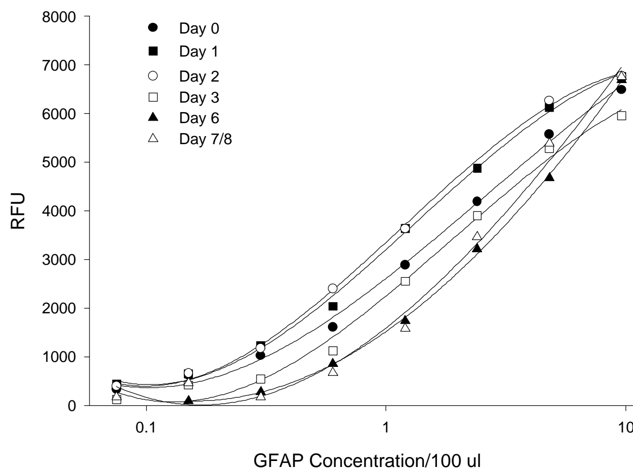

Figure 2. GFAP Standard was stored as a 320 ng/100 ul stock solution for a varying amount of time at 4°C. GFAP concentration starts to decline after storage for more than two days. RFU = Relative florescence units

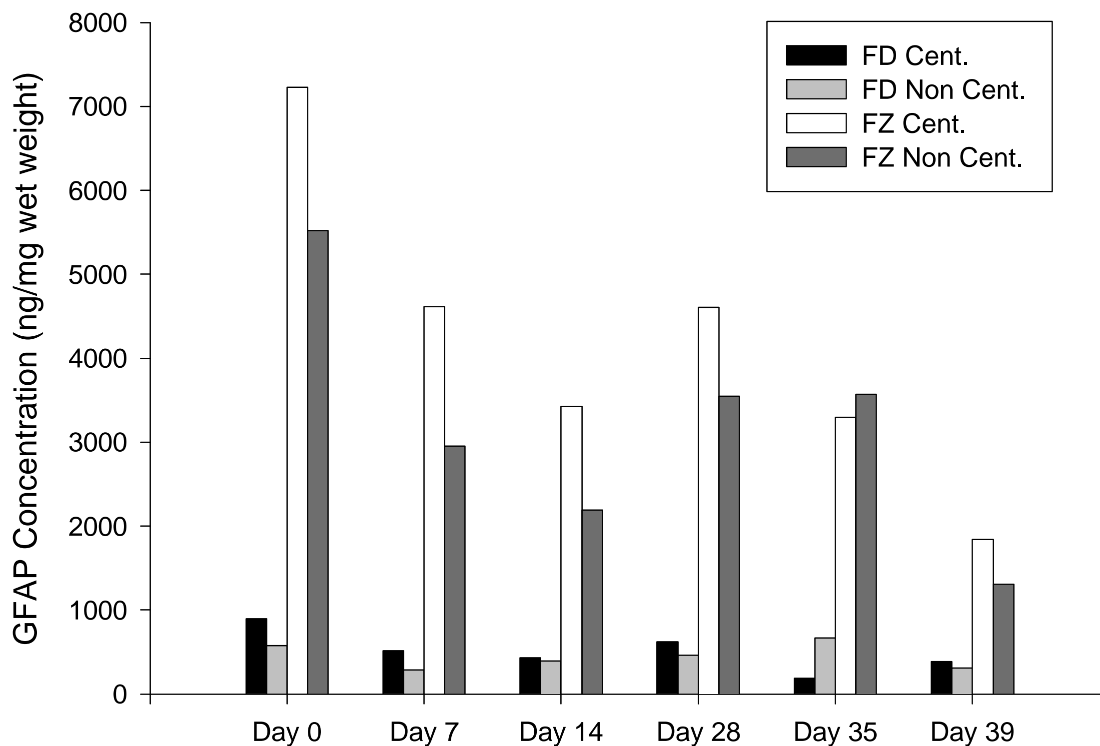

Figure 3. Comparison of centrifuged versus non-centrifuged freeze-dried (FD) and frozen (FZ) spinal cord homogenates on various days of storage at -20°C.