Development of Immunoassay for Detection of Meat and Bone Meal in Animal Feed

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ABSTRACT

An immunoassay system was developed for efficient detection of prohibited meat and bone meal (MBM) in animal feed. Monoclonal antibodies (MAbs) were raised against bovine smooth muscle autoclaved at 130°C for 20 min. Among the 1,500 supernatants of hybridoma cells screened, MAbs 3E1, 1G3, and 3E10 were selected and characterized in this study. The first set of MAbs produced, 3E1 and 1G3, had stronger reactivity against MBM than against smooth muscle that was heat treated at 90°C for 10 min. However, reactivity gradually increased against smooth muscle that was autoclaved at 130°C for up to 1 h. The enzyme-linked immunosorbent assay for detection of MBM in animal feed was optimized with the MAb 3E10 because of its superior performance. MAb 3E10 diluted to 100-fold was used to differentiate bovine MBM from that of other species in ingredients used for commercial animal feeds and could detect down to 0.05% MBM mixed in animal feed.

Bovine spongiform encephalopathy (BSE; also known as mad cow disease) is a fatal degenerative disease that affects the central nervous system of cattle (3). The onset of clinical signs is often associated with stress (herd change), calving, or estrus (6). The average age of onset of clinical signs is 5 years. According to epidemiological inquiries, meat and bone meal (MBM) contaminated with a transmissible spongiform encephalopathy agent is the primary vehicle of BSE transmission (4). MBM is produced by rendering the discarded fat, bones, offal, and whole carcases of cattle, sheep, pigs, and poultry. Although the use of MBM in cattle feed as a nitrogen supplement had been a common practice for several decades, changes in rendering operations in the 1970s and 1980s may have allowed the survival of the contagious agents and their transmission to cattle through MBM. To prevent the spread of BSE, the European Union banned the inclusion of ruminant-derived proteins in animal feed in 1988 (3).

The cattle industry is one of the largest agricultural industries in the United States; the estimated number of cattle and calves was 103.9 million head in 2003 (21). Annually, the United States produces 25.7 billion pounds (11.67 billion kilograms) of beef, and approximately 20 to 30% of the weight is discarded and used to produce MBM. The production of MBM was just below 4.2 billion pounds (1.91 billion kilograms) in 2000, worth about $360 million (19). In 1997, the U.S. Food and Drug Administration (FDA) introduced a regulation (21 CFR 589.2000) that prohibits the use in ruminant feed of proteins derived from mammalian tissues (24). According to this FDA regulation, the prohibited products include MBM, meat meal, bone meal, blood meal, tankage, and offal. Gelatin, milk products, or protein products from other species, such as pigs and horses, are exempt from this regulation. However, the lack of adequate control methods and of a rapid, sensitive, and reliable technique to specifically detect and quantify ruminant proteins hinders efficient implementation of these feed ban regulations (19).

Technical difficulties in developing the assay system have been encountered because of hydrolysis problems associated with the rendered MBM proteins that result from high-temperature and/or high-pressure treatment. The standard rendering condition used to produce MBM is steam treatment at 133°C for 20 min at a pressure of 300 kPa (7). It has often been reported that virtually all of the MBM proteins are degraded, thus making it difficult to identify a unique biomarker (12). Thus, the physical status of the sample imposes significant challenges for developing appropriate analytical methods for MBM detection. Among the currently available analytical methods for detection of protein, DNA, and bone fragments, immunochemical assays have been considered screening methods in terms of their practicability as long as sensitivity and specificity requirements are fulfilled (10). In this study, monoclonal antibodies (MAbs) were raised against autoclaved bovine smooth muscle that had been subjected to treatment similar to that used for MBM production. The affinity of these antibodies against autoclaved smooth muscles was tested to evaluate whether the antibody could effectively detect the rendered proteins. MAb 3E10 was selected because of its superior performance and used to develop an enzyme-linked im-

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munosorbent assay (ELISA) system, and the specificity and sensitivity of this system for detecting MBM mixed in animal feed were evaluated.

**MATERIALS AND METHODS**

**Production of the MABs.** Thermostable bovine intestine proteins were extracted from bovine smooth muscles. Fresh intestines were collected from a local processor immediately after slaughtering. The intestine samples were washed with ice-chilled water containing 0.25 mM phenylmethylsulfonyl fluoride (PMSF) and 0.75 mM benzamidine and prepared by removing the fat and inside mucous lining (18). The treated intestines were then frozen in liquid nitrogen, ground in a blender (Waring, Torrington, Conn.), and stored at −80°C. The sample powder (20 g) was homogenized in 120 ml of extraction buffer (300 mM KCl, 1 mM EGTA, 0.5 mM MgCl2, 0.25 mM PMSF, 0.01% NaN3, 1 mM benzamidine, and 50 mM imidazole–HCl, pH 6.9) with a Polyclon (Brinkmann Instruments, Westbury, N.Y.). The crude extracts were centrifuged at 40,000 × g for 30 min. For the heat treatment, the supernatants of crude extracts were incubated at 90°C for 10 min, centrifuged at 40,000 × g for 30 min, and then autoclaved at 130°C for 20 min, and the stability of the protein was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (16). To evaluate the reactivity of antibody with the smooth muscles rendered under conditions similar to those used for MBM production, the supernatants were also autoclaved at 130°C for 10, 30, 60, and 120 min. The protein concentrations of the prepared extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.) using bovine serum albumin (BSA; Sigma, St. Louis, Mo.) as the standard.

The autoclaved smooth muscle extract was used for immunization. MABs were produced at the Auburn University Hybridoma Facility according to the standard method of Kohler and Milstein (15) with modification. Three BALB/c mice were immunized with antigens (1 mg/ml) mixed with Freund’s complete adjuvant (1:1, vol/vol). Subcutaneous injections of antigens (1.0 mg/ml) mixed with Freund’s incomplete adjuvant were carried out at 3- to 4-week intervals. For hybridoma production, the lymphocytes collected from the spleen of an immunized mouse were fused with Ag8 myeloma cells (ATCC CRL 1580) using a polyethylene glycol fusion medium and then plated on 96-well plates in aminopterin-hypoxanthine-thymidine medium with peritoneal macrophage feeder cells. The cells were cultured in a CO2 incubator at 37°C. The supernatants of cell cultures were screened with the ELISA for the presence of antibodies capable of binding to heat-treated bovine smooth muscle extract and MBM. The hybridomas of interest were cloned by limiting dilution and extended. The isotypes of the MABs were determined by a mouse monoclonal antibody isotyping kit (Sigma) according to the manufacturer’s instructions.

**Preparation of test antigens.** Test antigens were prepared for the screening of hybridoma cells and to determine specificity and sensitivity of antibodies. Bovine and porcine MBM, animal feeds, and individual ingredients of the commercial animal feeds were directly obtained from commercial processing companies such as Cargill (Minneapolis, Minn.) and TBC (Birmingham, Ala.). To evaluate the sensitivity of the assay system, test samples were prepared by mixing different amounts of MBM (0.05, 0.1, 0.5, and 5%) with animal feed. Each sample (20 g) was homogenized in 60 ml of the extraction buffer and processed as described. The protein concentrations of the extracts were determined using the Bio-Rad protein assay with BSA as the standard.

**ELISA.** The ELISA was used to screen hybridoma cells capable of reacting with extracts of bovine smooth muscle and MBM and to evaluate cross-reactivity of MABs 3E1 and 1G3 (13). Antigens (2 μg per well) were coated on the 96-well plate (BD Falcon, San Jose, Calif.) overnight at 4°C, and then plates were washed three times with washing buffer (0.1% Tween 20 in phosphate-buffered saline [PBS], pH 7.4). The plate was then blocked with 1% BSA in PBS for 1 h. The test MABs were added to the wells, the plates were incubated for 2 h, and then the test MABs were bound with the secondary antibody, goat anti-mouse immunoglobulin M– or immunoglobulin G–alkaline phosphatase conjugate (1:3,000; Sigma), for 1 h. The substrate, p-nitrophenylphosphate (Sigma), was added, and the color development was quantitatively measured at 405 nm using an ELISA reader (Bio-Rad).

The ELISA was optimized to develop an analytical method with selected antibodies obtained from the second fusion. The plates were blocked with different blocking agents, such as BSA, gelatin, and milk protein. Tested concentrations for these blocking agents were 1, 3, and 5%. The MAB concentration was adjusted depending on the reactivity of the individual MAB. Two detergents (Triton X-100 and Tween 20, 0.1%) were added to PBS for the dilution of antibody to reduce nonspecific binding activity. For the secondary antibody, two different concentrations (1,000- and 3,000-fold) were tested. The optimized running condition was further used to test specificity and sensitivity of MAB 3E10. The assays were performed in duplicate and replicated three times.

**Analyses by SDS-PAGE and Western blot.** SDS-PAGE was carried out using a Mini-PROTEIN II electrophoresis cell (Bio-Rad) according to Laemmli (16). Total proteins were separated on the 10% separating gel and 4% stacking gel. Gels were stained with Coomassie brilliant blue G-250 (Bio-Rad). For the Western blot, the separated proteins in the gel were electrotransferred into a nitrocellulose membrane (Bio-Rad) with a semidry blotter unit (Amersham Biosciences, San Francisco, Calif.) at 0.8 mA/cm2 for 40 min using Towbin’s buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol). The blot was blocked in 5% BSA for 1 h followed by 2 h of incubation with MABs diluted to appropriate ranges (13). MAB binding onto the blot was detected by the secondary antibody enzyme conjugate, goat antimouse immunoglobulin M–alkaline phosphatase conjugate (1:1,000; Sigma). The blot was developed by adding 10 ml of 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (Sigma).

**RESULTS**

**Screening and cross-reactivity of MABs 3E1 and 1G3.** Hybridoma cells obtained from the first fusion batch were primarily screened against bovine MBM and smooth muscle extracts by means of ELISA and Western blot. In general, more hybridoma test cell lines had ELISA signals against MBM extract than against smooth muscle extract. Two MABs, 3E1 and 1G3, reacted to heat-treated MBM and intestine samples but exhibited no reactivity against BSA and animal feed on Western blots (data not shown). The specificity of antibodies was further tested by ELISA with individual ingredients used for commercial animal feeds (Fig. 1). The antibodies had stronger binding to MBM than to heat-treated bovine intestine at 90°C for 10 min. Although both MABs were not able to differentiate bovine MBM from porcine MBM, they were relatively specific for discriminating most ingredients used for commercial animal
Reactivity of MAbs 3E1 and 1G3 with autoclaved bovine smooth muscles. To evaluate the reactivity of antibody with the autoclaved bovine smooth muscles treated under conditions similar to those used for MBM production, the antibodies diluted 20-fold were tested by ELISA with variously heat-treated bovine intestine samples, i.e., crude, heated at 90°C for 10 min, and autoclaved at 130°C for 10, 30, 60, and 120 min (Fig. 2). MAb 3E1 and 1G3 did not react with intestine samples that had not been heat treated. However, heat treatment of bovine intestines greatly affected their reactivity with both antibodies. These antibodies produced stronger signals with the bovine intestine samples autoclaved at 130°C than with the intestine that was heat treated at 90°C for 10 min. MAb 3E1 had a slightly stronger signal with the smooth muscle autoclaved at 130°C for 30 min than with MBM. The strongest reactivity of MAb 3E1 was with smooth muscle autoclaved for 60 min. With MAb 1G3, the ELISA signal remained unchanged for samples autoclaved for up to 120 min.

The specific affinity of MAb 3E1 for the blotted bands of thermostable proteins in autoclaved samples was detected on Western blot (Fig. 3). The presence of an intact thermostable protein corresponding to approximately 150 kDa...
FIGURE 4. Cross-reactivity as determined by ELISA of Mab 3E10 at 100- and 250-fold dilutions with ingredients used in commercial animal feed. The assay was replicated three times.

**TABLE 1. Sensitivity of Mab 3E10 at 100-fold dilution for detecting MBM mixed in animal feed**

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Sensitivity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBM in animal feed (%)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.204 ± 0.143</td>
</tr>
<tr>
<td>5</td>
<td>0.545 ± 0.047</td>
</tr>
<tr>
<td>0.50</td>
<td>0.440 ± 0.033</td>
</tr>
<tr>
<td>0.10</td>
<td>0.341 ± 0.069</td>
</tr>
<tr>
<td>0.05</td>
<td>0.224 ± 0.043</td>
</tr>
<tr>
<td>0</td>
<td>0.113 ± 0.026</td>
</tr>
<tr>
<td>BSA</td>
<td>0.144 ± 0.038</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.129 ± 0.035</td>
</tr>
</tbody>
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$^a$ Mean ± standard deviation optical density at 405 nm.

was detected following staining with Coomassie blue R-250 in extracts heat treated at 90°C for 10 min and in extracts autoclaved for 10 and 30 min. The antibody did not react with samples that were not heat treated but did react with MBM. A smeared band produced from degradation of protein in the MBM extract was detected by immunostaining with Mab 3E1. Gradual degradation of proteins by thermal treatment was detected in the autoclaved smooth muscle extracts following staining by Coomassie blue R-250 or immunostaining with Mab 3E1. However, the antibody clearly detected the blotted band corresponding to the 150-kDa protein in the smooth muscles autoclaved for up to 60 min. Mab 3E1 obviously bound with degraded proteins from the smooth muscle extract treated for 2 h.

**Optimization of ELISA with Mab 3E10.** More positive hybridoma cells capable of binding with bovine MBM and intestine were screened from the second fusion batch than from the first batch. Among the 700 hybridoma cells screened, nine samples produced very strong ELISA signals against bovine MBM (optical density at 405 nm [OD$_{405}$] > 3.0). The class of Mab obtained in this study was all immunoglobulin M. Positive cells were immediately cloned and further tested with more antigens to optimize ELISA conditions for specific detection of MBM in animal feed. Of the tested antibodies, Mab 3E10 had ELISA patterns of affinity against autoclaved smooth muscles similar to those observed with Mabs 3E1 and 1G3, thus indicating the potential of Mab 3E10 for use in the development of assay systems. To achieve optimization, the antibody was tested in the ELISA under various conditions, such as different dilutions, dilution buffers, and blocking agents. Dilution of Mab 3E10 100-fold in PBS was most efficient for all the parameters. The affinity of Mab 3E10 was greatly affected by the tested blocking agents, such as BSA, gelatin, and milk protein (data not shown). Among these agents, 1% gelatin was most successful in decreasing non-specific binding interference. Mab 3E10 had a very strong signal (OD$_{405}$ > 3.0) at 100-fold dilution for MBM and bovine MBM, but a negligible signal (OD$_{405}$ < 0.11) for BSA, gelatin, and milk protein. Dilution of the secondary antibody at 3,000-fold was suitable for the assay with Mab 3E10.

**Cross-reactivity and sensitivity of 3E10 in detecting MBM in animal feed.** The cross-reactivity of Mab 3E10 was tested with 15 different ingredients used in commercial animal feed production (Fig. 4). The antibody at 100-fold dilution had a strong signal with bovine MBM (OD$_{405}$ > 3.0). This antibody also could differentiate bovine antigens from those of other species, such as porcine MBM and chicken intestine. Mab 3E10 cross-reacted with canola pellet and wheat. The antibody did not react with most of the plant protein and other ingredients tested, such as high-fat rice bran, dried egg, blood meal, and dried porcine plasma. Therefore, this antibody was considered promising for distinguishing bovine MBM from other ingredients allowed in animal feed.

The sensitivity of the assay system was evaluated by testing Mab 3E10 with different amounts of MBM (0 to 5%) mixed into animal feed (Table 1). The antibody diluted 100-fold detected MBM down to 0.05% in animal feed. When the ELISA signal was plotted against the tested MBM concentration, a linear response was obtained from Mab 3E10 ($r^2 = 0.971$).

**DISCUSSION**

Thermostable proteins have been used to raise monoclonal or polyclonal antibodies for the detection of prohib-
ited MBM or other ruminant proteins mixed in animal feeds (2, 5). However, protein degradation resulting from the stringent rendering conditions for MBM production has made it difficult to identify a biomarker for the development of adequate assay systems. Using gel electrophoresis, Hofmann (12) detected no sharp or discrete protein bands with test MBM, indicative of the damage to protein integrity. The detection rate of the assay system was greatly dependent on the temperature used for product treatment, even though the antibodies used for the tests were raised against heat-stable proteins (25). These findings indicate the need for using heat-resistant biomarkers to raise feasible antibodies.

In our previous study, the presence of a thermostable protein corresponding to approximately 150 kDa was detected by SDS-PAGE in bovine MBM and heat-treated intestine muscle (13). The identified biomarker was a high-molecular-weight caldesmon (h-caldesmon) that is present in all types of smooth muscle but is absent from cardiac and skeletal muscle (9). h-Caldesmon contains four or five alpha helices of short to intermediate length in the N-terminal domain and a long continuous alpha-helix with at least nine repeats of a 12-residue unit, KAEEE(K/R)KAAEEK, in the central domain (14). The peculiar property of the alpha-helical content in h-caldesmon contributes to its heat stability (11). MAB 5E12 raised against purified h-caldesmon had stronger binding affinity against smooth muscle extract autoclaved at 130°C than to the extract heated at 90°C (14). This antibody produced a slightly weaker ELISA signal with the smooth muscle autoclaved at 130°C for 30 min than with MBM. Therefore, in this study, autoclaved smooth muscle was selected to raise sensitive and specific antibodies against MBM mixed in animal feed.

Antibodies produced against the extract of autoclaved smooth muscle had strong binding affinity for smooth muscle extracts autoclaved at 130°C for up to 1 h. In particular, MAB 3E1 had specific affinity with the blotted band corresponding to h-caldesmon in autoclaved smooth muscle on Western blot, as previously observed with MAB 884 (13). We found that the fortified thermostable biomarker in heat-treated smooth muscle could induce strong antibody responses for these MBM-specific antibodies and that the biomarkers in autoclaved smooth muscles were better for raising antibodies than were those in MBM extract, which failed to induce strong antibody responses (14).

A few immunological methods for the detection of MBM have been reported, but bovine-specific antibodies were not developed. In this study, MABs 3E1 and 1G3 were of limited value in differentiating bovine MBM from porcine MBM and cross-reacted with gelatin and related products, as found for MAB 5E12 (13). However, the use of gelatin as a blocking agent greatly reduced the cross-reactivity with gelatin and other associated products and improved specific detection of bovine MBM with MAB 3E10. This antibody was also suitable for the sensitive and quantitative detection of MBM in animal feed. The sensitivity of MAB 3E10 (0.05%) met the detection limit of 0.1% MBM in animal feed suggested by the FDA (19). The sensitivity of the reported ELISA with a polyclonal antibody was 1 to 5% MBM in animal feed (2). The antibody prepared by Chen et al. (5) against a heat-stable protein (troponin I) had a detection limit of 0.5% MBM in animal feed.

Because of problems related to protein hydrolysis, immunoassay systems rarely have been applied for the detection of MBM. Because DNA is more stable than proteins, PCR assays for specific amplification of mitochondrial DNA are more frequently used than immunoassay for the development of analytical methods (20). However, Frezza et al. (8) found a drawback to the assay system associated with DNA degradation and suggested that only a short amplicon (147 bp) could be used for efficient detection of bovine DNA residues in MBM treated under the conditions currently stipulated by European regulations (7, 17). The critical drawback of the PCR assay system is the lack of differentiation between allowable and prohibited bovine materials, as indicated by generation of a positive signal with milk and even fat (19). The PCR system also requires expensive equipment, reagents, and well-trained staff and is not practical for field testing.

With the announcement of the first outbreak of BSE in the United States (23), it became important to quickly develop a routine field method for monitoring MBM contamination of animal feeds so that regulations aimed at eradicating BSE could be enforced. In this study, the immunoassay with MAB 3E10 performed well for the detection of MBM; it is simple, specific, sensitive, and adaptable to either field or laboratory settings.

MABs prepared against smooth muscle extract treated at 130°C for 20 min were useful in detecting MBM and autoclaved smooth muscle proteins. MAB 3E10 allowed sensitive and quantitative detection of MBM mixed in animal feed at 0.05%, indicating the potential for use of this MAB in developing a routine analytical method for the detection of MBM in animal feed.

REFERENCES


