CHAPTER 17. IDENTIFICATION OF ANIMAL SPECIES IN COOKED AND CANNED MEAT AND POULTRY PRODUCTS

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17.1 Introduction

This chapter describes a procedure which can identify the species of animal tissue used as ingredients in cooked and canned meat foods. A simple water extraction of samples along with an amplified double antibody sandwich type enzyme-linked immunosorbent assay (ELISA) is employed. With this type of ELISA, capture antibody is bound to the polystyrene plastic of microplate wells. If tissue antigens are present, they are bound by solid phase, species specific, capture antibody in the wells. After washing to remove unbound material, a biotinylated antibody with the same specificity as the capture antibody is added. Biotin is a vitamin which can be covalently bound to antibody with relative ease and with virtually no deleterious effect on the specificity of the antibody molecule. The biotinylated antibody is bound to the solid phase only if antigens of the species in question were previously captured; otherwise the unbound biotinylated antibody is removed by washing. Streptavidin-Horseradish peroxidase conjugate is then added to the wells. Streptavidin is a glycoprotein which has a remarkable affinity for biotin ($K_D = 10^{-15}M^{-1}$). Any biotin in the solid phase complex will bind the streptavidin-enzyme conjugate. Unbound conjugate is removed by washing and a substrate for the enzyme is added. If the species antigens were present in the test sample, a green color will develop as a result of the action of the bound enzyme on the substrate. This assay allows for high sample output and same day results. The procedure must be repeated for each species in question using the appropriate species specific antibodies.

Antigenic molecules, when subjected to high temperatures (such as those encountered in the production of cooked and canned meat products) will denature. The denaturing process includes a randomization of the tertiary structure and reduced solubility. These changes cause a concomitant reduction in the molecules' antigenicity. Hence, standard immunoassays are usually not possible when samples have been subjected to denaturing heat.

For development of the present assays, unheated skeletal muscle tissue was fractionated and antigenic molecules were isolated which proved to be relatively heat stable. These antigens are highly

soluble, acidic glycoproteins. Antisera prepared to them are species specific and are reactive with extracts of cooked and canned meat products containing that species.

Important

One should not attempt to quantify the amount of a particular species tissue in a sample based on this assay. Because the antigens are not completely heat stable, a higher temperature and longer cooking time will result in a lower reaction. Therefore the intensity of the reaction is related to the sample processing as well as to the level of antigen present.

A slight cross reaction occurs among red meat species. The colorometric detection system of the ELISA does not allow distinction between a low level homologous reaction and a cross reacting species. This situation has been addressed by establishing an absorbance value which must be exceeded for a sample to be considered positive. The selected absorbance value is significantly higher than any cross-reaction or background color. DO NOT test raw products by this assay, as the cross reactions with raw tissue extracts might exceed the cutoff value.

17.2 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC plate reader; #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer; #78-431-00.
c. Flow (ICN) Vacuum pump for above washer; #78-426-00.
d. Flow Titertek Multichannel pipette; 8 channel, adjustable 50-200 ul volume; #77-859-00.
e. Eppendorf repeater pipette (Daigger Sci. Co. #G20551) with accessory of 1.25 and 2.5 ml capacity combitips, and a 0-200 µl variable pipette with tips.
f. Flow (ICN) Laboratories, Linbro EIA II Plus plates, flat bottom, 96 wells #76-181-04 and covers.
g. Flow (ICN) Plate Sealers #76-401-05.
h. Stomacher®, Model 80 (Tekmar Co., Cincinnati, Ohio).
i. Whirl-pak® bags; 6 oz. and 18 oz. sizes.
j. Centrifuge, capable of operation at 15,600 G (Eppendorf, Model 5412; Brinkman Instruments, Inc.), and appropriate centrifuge tubes.
k. Refrigerator (4°C)/Freezer (-20°C).
l. Aluminum foil.
m. Erlenmeyer flasks, 125 ml.

n. High humidity chamber (air-tight plastic container).
o. Disposable Millipore® filters, 0.45 µm, luer lock.

17.21 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, S-374).
b. NaH_2PO_4 (Fisher, S-369).
c. NaCl (Fisher, S-271).
d. Citric acid, anhydrous (Fisher, A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, H-323).
f. Tween 80 (Fisher, T-164).
g. ABTS substrate indicator; 2,2'azino-di-(3-ethyl benzthiazole sulfonic acid), (Sigma, A-1888).
h. Thimersol (merthiolate) (Sigma, T-5125)
i. TRIZMA Base (Sigma, T-1503).
j. TRIZMA HCl (Sigma, T-3253).
k. HCl (Baker, I-9535).

17.22 Reagents Supplied By Quality Assurance Branch, MD, Athens, GA To TSL's

a. Coating antibody, (store at -20°C or below).
b. Biotinylated antibody, (store at -20°C).
c. Streptavidin-Horseradish peroxidase conjugate, (store at 4°C).
d. Normal rabbit serum, (heat inactivated at 56°C for 1 h, filter sterilized; store at -20°C or below).

17.23 Preparation of Stock Solutions

a. Coating buffer (0.05 M TRIS-HCl pH 7.7)

Weigh out 5.72 g TRIZMA-HCl and 1.66 g TRIZMA-BASE and 0.10 g thimersol (merthiolate); dissolve and bring to 1 liter with distilled water.

b. Wash buffer (PBST; 0.075 M phosphates, 0.075 M NaCl and 0.05% Tween 80, pH 7.2)

Weigh out 15.78 g Na_2HPO_4 and 5.37 g $NaH_2PO_4.H_2O$ and 8.76 g NaCl; dissolve in distilled water and add 1 ml Tween 80. After the Tween 80 dissolves, bring the volume to 2 liter with distilled water.

c. Diluent (PBST containing 10% heat inactivated normal rabbit serum)

Prepare this solution the same day it will be used. Add 1 ml of heat inactivated normal rabbit serum to 9 ml of wash buffer. This will provide sufficient volume to run one complete plate.

d. Normal saline

Weigh out 8.5 g NaCl; dissolve and bring to 1 liter with distilled water.

e. 2,2'azino-d-3-ethylbenzthiazoline sulfonic acid (ABTS)-H_2O_2 Substrate (pH 3.9)

Weigh out 0.904 g citric acid (anhydrous), 0.750 g Na_2HPO_4, and 22 mg ABTS; dissolve in distilled water; add 15 µl of 30% H_2O_2 and bring the volume to 100 ml with distilled water. Filter sterilize and store in the dark.

f. Stop solution (0.1 M citric acid)

Weigh out 1.92 g citric acid (anhydrous); dissolve and bring to 100 ml with distilled water.

g. Controls

Dice 20 g of lean, raw skeletal muscle tissue and place in an 18 oz. Whirl-Pak® bag, along with 60 ml of normal saline. Place the bag and contents into a stomacher and stomach for 10 seconds, then let stand for one hour at room temperature. Place the contents of the bag into a 125 ml Erlenmeyer flask, seal with aluminum foil and place in a boiling water bath for 15 minutes. Transfer to centrifuge tube(s), and centrifuge at 10,000 x G for 15 minutes. Filter the supernatant through a 0.45 µm filter, dispense in 0.5 ml aliquots and store at -20°C. Repeat this procedure for each species.

17.3 Sample Extraction

Prepare sample extracts from cooked or canned meat products as follows:

a. Place 5 g of diced sample in a 6 oz., Whirl-pak® bag along with 10 ml distilled water.

b. Place bag and contents into a stomacher and stomach for 60 seconds.

c. Remove from stomacher and leave undisturbed for one hour at room temperature.

d. Pour off some of the liquid into a centrifuge tube and centrifuge at 15,600 x G for 10 minutes.

e. The clear supernatant tissue extract is used in the ELISA.

17.4 Coating Microelisa Plates with Solid Phase Antibody

a. Obtain five flat bottom, 96 well, Linbro EIA II plus plates and plate sealers from stock supplies.

b. Prepare coating antibody solution of the desired species by adding 100 µl of the anti-species coating antibody to 50 ml of coating buffer. Mix by occasional gentle swirling over a one hour period.

c. Using the 8 channel pipette, place 100 µl of the coating antibody solution into all wells of the microelisa plates.

d. Seal each plate and mark with the species and the date on the sealers. Place the plates in a high humidity chamber and store at 4°C for a minimum of 24 h. These plates can be used in the ELISA for up to six months unless the coating antibody solution has desiccated.

17.5 Performance of the ELISA

a. Remove an antibody sensitized plate with the desired species specificity from the high humidity chamber and remove the plate sealer.

b. Place the plate on the carrier of the microplate washer, which has been primed with wash buffer and set to deliver 300 µl buffer to each well. Wash and aspirate the plate three times.

c. Invert the plate and rap three times onto a soft paper towel placed on the lab bench to remove remaining liquid from the wells.

d. Place 100 µl of normal saline into wells 1A through 1H for blanks.

e. Place 100 µl of positive control tissue extract into wells 2A through 2D, (use the species being tested for; if testing for poultry, use chicken as the positive control).

f. Place 100 µl of the negative control tissue extract into wells 2E through 2H, (use the same species as on the product label. If more than one species on label, additional sets of 4 wells per species are used, starting with column 3. If no species on label, then any heterologous red meat species can be used).

g. All remaining wells are used for samples. Samples should be run in replicates of 4 wells each, 100 µl per well. Avoid transferring any fat from the sample extracts to the wells.

h. The plate is covered and left at room temperature for 45 minutes.

i. Wash wells by repeating steps (b) and (c).

j. Dilute the biotinylated antibody to the proper working dilution in the diluent (freshly prepared PBST containing 10% NRS). The proper working dilution of the biotinylated antibody for each species is previously determined by performing the procedure described in Section 17.7.

k. Place 25 µl of diluent into the bottom of wells 1A through 1H.

l. Place 25 µl of the properly diluted biotinylated antibody into the bottom of each well, except wells 1A through 1H. Observe that the bottom of each well is covered with liquid. If not, gently tap the edge of the plate until this is accomplished. Avoid getting any antibody hung up on the sides of the wells.

m. The plate is covered and left at room temperature for 45 minutes.

n. Wash wells by repeating steps (b) and (c).

o. Dilute the Streptavidin-horseradish peroxidase conjugate by first making a 1:41 dilution (e.g. 25 µl plus 1 ml of the diluent), then make a 1:50 dilution (e.g. 100 µl of 1:41 plus 4.9 ml of the diluent) for a total dilution of 1:2050.

p. Place 25 µl of the diluted conjugate into the bottom of each well. Again observe that the bottom of each well is covered with liquid and that none of the conjugate is hung up on the sides of the wells.

q. The plate is covered and left at room temperature for 30 minutes.

r. Repeat step b. except set the microplate washer to wash 4 times. Wash and aspirate the plate four times.

s. Repeat step (c).

t. Place 50 µl of ABTS-H_2O_2 substrate solution into the bottom of all wells.

u. The plate is covered and left at room temperature for 30 minutes.

v. Place 50 µl of stop solution (0.1M citric acid) into all wells.

w. Program the "warmed-up" Multiskan plate reader for the current date, Mode 2, reference filter #4 (492 nm), absorbance filter #2 (414 nm), and blank the instrument on column 1. If a Bio-Tek plate reader is available for use, program the "warmed-up" Bio-Tek plate reader for the current time, date and program options. The option selection on this instrument gives instructions to allow you to select dual wavelengths, filter #1 to 415 nm, filter #2 to 490 nm, all print options, and to indicate that all first column wells are blank.

x. Press the start button and obtain a printed copy of absorbance values for each well.

17.6 Data Analysis

a. Determine the mean value of the positive control wells (2A through 2D) and the standard deviation.

b. Determine the mean value of the negative control wells (2E through 2H).

c. The test is valid if the mean of the positive controls is greater than 0.60, the standard deviation is not more than 0.06 and the mean of the negative controls is less than 0.06. If otherwise, the test is invalid, and should be repeated.

d. Determine the mean value for each sample and its' standard deviation. Any sample whose mean absorbance value, minus three standard deviations, is greater than 0.25 will be considered positive; all other samples are negative.

e. Report a positive result as follows: "(Species*) antigens were detected in this cooked or canned meat product by an enzyme-linked immunosorbent assay."

Report a negative result as follows: "(Species *) antigens were not detected in this cooked or canned meat product by an enzyme-linked immunosorbent assay."

* Substitute the name of the species which was tested for.

17.7 Determination of Proper Working Dilution of Biotinylated Antibody for Each Species. (Section contributed by Mark S. Dreyfuss)

This section describes a procedure for determining the proper concentration of biotinylated antibody to be used for performing the formal ELISA procedure described in Section 17.5. This is necessary to insure the proper balance of reagents within this ELISA system's parameters to achieve the desired sensitivity.

a. Remove an antibody sensitized plate with the desired species specificity from the high humidity chamber and remove the plate sealer.

b. Perform steps b., c. and d. of Section 17.5 (Performance of the ELISA) in the manner described.

c. Place 100 µl of positive control tissue extract (100%) into wells A through D in each column numbered 2 through 6 (use the species being tested for; if testing for poultry, use chicken as the positive control).

d. Place 100 µl of a 2% positive control tissue extract (1:50 dilution in normal saline of the same 100% positive control tissue extract used above) into wells A through D in each column numbered 7 through 11.

e. Place 100 µl of the negative control tissue extract into wells E through H in each column numbered 2 through 6 (any *heterologous* red meat or poultry species can be used).

f. The plate is covered and left at room temperature for 45 minutes.

g. Wash wells by performing steps b. and c. of Section 17.5 (Performance of the ELISA) in the manner described.

h. Dilute the biotinylated antibody in the diluent (freshly prepared PBST containing 10% NRS) and place in the respective plate wells as follows:

i. Determine five appropriate dilutions to make the biotinylated antibody. This is dependent upon the recommended working dilution of the antibody by the supplier or manufacturer. If the dilution recommended is high, ex: 1/400, use increments of 1/50. If the dilution recommended is low, ex: 1/60, use increments of 1/10.

ii. Make each dilution separately with PBS-Tween-10%NRS diluent in a separate test tube.

iii. Place 25 µl of diluent into the bottom of wells 1A through 1H.

iv. Place 25 µl of the first determined diluted biotinylated antibody into the bottom of each well of column 2 and wells A through D of column 7. Observe that the bottom of each well is covered with

liquid. If not, gently tap the edge of the plate until this is accomplished. Avoid getting any antibody hung up on the sides of the wells.

v. Place 25 µl of the second determined diluted biotinylated antibody into the bottom of each well of column 3 and wells A through D of column 8.

vi. Place 25 µl of the third determined diluted biotinylated antibody into the bottom of each well of column 4 and wells A through D of column 9.

vii. Place 25 µl of the fourth determined diluted biotinylated antibody into the bottom of each well of column 5 and wells A through D of column 10.

viii. Place 25 µl of the fifth determined diluted biotinylated antibody into the bottom of each well of column 6 and wells A through D of column 11.

i. Proceed to complete the above assay by performing steps m. through x. of Section 17.5 (Performance of the ELISA) in the manner described.

j. Analyze the data by determining the mean value and standard deviation of each identical set of 4 wells for the 100% positive control, the 2% positive control and the negative control for each of the 5 respective biotinylated antibody dilutions tested. If any negative control means are greater than an OD value of 0.06, then this determination is invalid and must be repeated. Any mean whose standard deviation is greater than 0.06 is invalid and must be repeated.

k. The lowest dilution of biotinylated antibody which produces a mean OD value most closely equal to but not less than 0.6 for the 100% positive control wells, AND (for the same dilution) also produces a mean OD value greater than 0.25 for the 2% positive control wells is the proper working dilution of the biotinylated antibody to be used in the formal assay.

l. The working dilution of the biotinylated antibody determined above should then be used each time that this stock bottle of concentrated biotinylated antibody is used for performing the formal ELISA for species

determination in cooked meat/poultry samples. When a fresh supply of the same lot of concentrated biotinylated antibody is received, this dilution determination should be verified. When a new lot of biotinylated antibody is received, then the entire determination should be performed.

m. This dilution value is good for only the tested species. Each species ELISA will need to have it's biotinylated antibody level determined separately. Record that value in a standard operating procedure notebook along with the date and signatures of the analyst and supervisor confirming the results.

17.8 Quality Control Procedures

a. The assay reagents have been standardized for use only with ICN Linbro EIA II Plus microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. The coating buffer solution (0.05 M Tris-HCl, pH 7.7) should be checked for proper pH before use and the stock solution should be made fresh after 6 months.

d. The proper concentration (dilution) of each species of biotinylated antibody to be used in the formal assay must be previously determined by performing the procedure described in Section 17.7. This is necessary to insure that the reagents are balanced within the performance parameters of the assay system to achieve the desired sensitivity.

e. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

f. Be sure the stock, commercial Streptavidin-peroxidase reagent has not deteriorated to the point of producing abnormally low final O.D. readings. Use only an unexpired lot of this reagent.

g. Do not use antibody coated plates that are more than 6 months old. Any stored plates which show desiccation of the antibody coating solution should be discarded.

h. Positive and negative controls must be used on each analytical run. The mean of the negative control must be less than 0.06 and the mean of the positive control must be greater than 0.60 with a standard deviation of no more than 0.06 for the particular test run to be considered as valid.

i. Positive and negative control extracts must be prepared from authentic, raw, skeletal, muscle tissue exactly as described in the procedure.

17.9 Selected References

Andrews, C. D., R. G. Berger, R. P. Mageau, B. Schwab, and R. W. Johnston. 1992. Detection of beef, sheep, deer, and horse meat in cooked meat products by enzyme-linked immunosorbent assay. J. Assoc. Off. Anal. Chem. Int. 75:572-576.

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