Counter immunoelectrophoresis: a simple method for the detection of species-specific muscle proteins in heat-processed products

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ABSTRACT: Counter immunoelectrophoresis (CIE) was used for the detection of species-specific muscle proteins in food products. This technique allowed the detection of pork, beef, poultry, or and kangaroo meats in heat-processed products at concentrations below 1.5%. CIE is based on the use of species-specific polyclonal antibodies prepared by immunisation of rabbits with heat-stable antigens extracted from visibly fat-free muscular tissue heated to 75°C, 100°C, or 120°C for 30 minutes. Adulterations in terms of declared product compositions were demonstrated by this method in 7 of the 50 tested commercial products.

Keywords: counter-immunoelectrophoresis; muscle protein; thermostable antigen; antiserum; heat-processed meat products

Identification of meat species is an important task of food quality control. Intentional adulteration of meat products with other-than-declared meat species can bring the manufacturer considerable economic profit. Moreover, adulteration is associated with a hazard of allergic reactions in sensitive consumers. The significance of meat species detection in meat products has risen dramatically in association with the emergence of bovine spongiform encephalopathy which necessitated the implementation of effective methods for checking the declared meat products composition.

Meat species can be identified by serological (Cutrufelli *et al.*, 1987; Reddy *et al.*, 2000), histological (Tremlova, 2000), immunochemical (Rencova *et al.*, 2000), or molecular biological methods (Matsunaga et al., 1999; Krcmar and Rencova, 2001).

The procedures of the identification of raw meat species by electrophoretic methods or ELISA are rather simple (Patterson and Whittaker, 1984). However most of the commercial antisera intended for species identification are prepared against blood proteins and are therefore suitable only for raw meat species differentiation

The difficulties in the preparation of species specific antisera against heat-processed proteins, as described by Kang'ethe and Lindqvist (1987), Kang'ethe and Gathuma (1987), and Hofmann *et al.* (1996) result from thermal denaturing of proteins. Therefore, antibodies to heat-stable soluble proteins, which retain their antigenicity after heating to 75°C, 100°C and even after autoclaving at 120°C for 30 min, must be prepared.

Such proteins are present especially in adrenal tissues (Milgrom *et al.*, 1963) and in small amounts also in striated muscles (Hofmann, 1977).

In our experiments like Patterson and Jones (1989), crude mixture of proteins that remained soluble after heat treatment was used as the antigen for immunisation. Hayden (1977), who immunised serum donors with chicken troponine was able to detect chicken protein mixed into beef with a sensitivity of 1%, 3%, and 5% by agar-gel difussion method. And also Hayden (1979) with the help of antisera against sheep, swine and horse myoglobine detected 3% level of adulteration in meat products heated to 70°C by agar-gel diffusion method again. However, the sensitivty decreased

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for 5 to 10% when heat-stable proteins of the adrenal gland were used for the immunisation (Hayden, 1981).

So far no generally applicable immunisation scheme has been suggested. Most of the authors prepared their antisera in rabbits (Swart and Wilks, 1982; Cutrufelli *et al.*, 1987; Martin *et al.*, 1988), but some of them, such as Mageau *et al* (1984) preferred goats and sheep. Sherikar *et al.* (1988) prepared anti-bovine sera by immunisation of the phylogenetically related buffalo calves. Reddy (2000) raised antisera in rabbits using native and heated testicular antigens from cattle, sheep, goat, or buffalo. To overcome the problem of absorption to make the antisera monospecific, antisera were raised also in phylogenetically related species. The sensitivity in testing of heat-processed products ranged from 10 to 20%.

To a certain extent, cross reactions can be avoided by saturation of antisera with the respective antigen(s) followed by centrifugation and/or affinity chromatography in CNBr-activated Sepharose 4 B (Martin *et al.*, 1988). Alternatively, the donor animals can be immunised with antigens purified by fractionation with ammonium sulphate (Berger *et al.*, 1988; Martin *et al.*, 1992. A review on cross-reactivity of commercial antisera was published by Pickering *et al.*,(1992).

The objective of our investigations was to develop a simple and reliable method based on the demonstration of small amounts of soluble muscle proteins, such as the low-molecular actin, tropomyosin (Chin-Sheng Cheng and Parrish,1979), and troponin (Sherikar *et al.*, 1993), which are present in extracts of heat-processed meat.

MATERIAL AND METHODS

Solutions

1. PBS (0.05M phosphate, buffered saline solution, 0.05 M, pH 7.2)
2. Tris-succinic acid (0.1M trishydroxymethylaminomethane with pH adjusted to 7.2 by 0.1M succinic acid)
3. Agarose (Lachema, Czech Republic)
4. Amidoblack 10B
5. Destaining solution (250 ml of petrol-denatured ethanol and 100 ml of concentrated sulphuric acid, completed with distilled water to 1 l)

Antigens

Antigens for immunisation were prepared from samples of visibly fat-free muscular tissues of the cattle, swine, chicken and kangaroo. The samples were homogenised in a blender with equal parts of 0.05M phosphate-buffered saline, pH 7.2 (PBS); the homogenates were heated for 30 min either in a water-bath at 75°C, or 100°C, or in an autoclave at 120°C, gauze-filtered and centrifuged at 10 000 × g and 4°C for 15 minutes. The protein content in the supernatants was determined using the Bicinchinonic Acid kit (Sigma). The antigen concentration was adjusted to 2 mg per ml.

Heterologous antigens for specificity tests of CIE were prepared by the same procedure from muscular tissues of cattle, chickens, turkeys, ostriches, horses, swine, goats and kangaroos.

Animals

New-Zealand White rabbits, three months old. Three rabbits for each type of antigen and each treatment temperature were used.

Immunisation procedure

For the first dose, the immunisation antigen was mixed 1 : 1 with complete Freund's adjuvant (Sigma, USA) and administered intradermally. The dose of 0.2 ml (0.4 mg protein) was applied to 10 sites on the back. The immunisation was repeated 28 days later when the same dose of the antigen completed with Al-Span-Oil adjuvant (USOL, Czech Republic) instead of Freund's adjuvant was administered subcutaneously at two sites. The latter procedure was repeated three times at 10-day intervals. The rabbits were bled by cardiac puncture after the last treatment and blood sera after blood-coagulation, separated by centrifugation at 1000 × g and 4°C for 20 min, were stored at –20°C.

Sensitivity of the method was done by CIE method using twofold dilution series of homologous antigen extracts within the range 100 to 0.5%. Four antisera (one for each animal species identification) according to the best sensitivity and specificity were chosen.

The antisera showed none or very weak cross-reactions which were eliminated by saturation with the cross-reacting antigen (1/10 of the antisera volume). Then the antiserum was centrifuged at 150 000 × g and 4°C for 2 h and the supernatant was used for specificity testing. The whole procedure was repeated when the cross-reactivity persisted.

Sample processing

Samples of 100 g of meat and meat products (salamis, frankfurters, cooked ham, sausages, pork and

beef in natural juice, canned ham, fresh meat, boiled pressed meat, canned sandwich spreads, and canned meat) were processed in a blender with 100 ml of PBS, gause filtered and the homogenate was centrifuged at 10 000 × g and 4°C for 15 min and the supernatant was used for analysis.

Counter-immunoelectrophoresis

Wells with a diameter of 4 mm were arranged in 15 ml of 1.5% agarose (Lachema, Czech Republic) gel in eight pairs on the right and the left side of the plate (0.5cm apart). The size of the plate was 8.5 × 8.5 cm.

Table 1. Antisera specificity

Antigen	°C	Antiserum			
		RAB 75/2	RASw 100/5	RACh 100/2	RAKa 100/7
B	75	+	–	–	–
	100	+	–	–	–
	120	+	–	–	–
Sw	75	–	+	–	–
	100	–	+	–	–
	120	+	+	–	–
H	75	–	–	–	–
	100	–	–	–	–
	120	–	–	–	–
O	75	–	–	–	+
	100	–	–	–	+
	120	–	–	–	–
Ch	75	–	–	+	–
	100	–	–	+	–
	120	–	–	+	+
T	75	–	–	+	–
	100	–	–	+	–
	120	–	–	+	+
Os	75	–	–	+	–
	100	–	–	+	–
	120	–	–	+	+
Ka	75	–	–	–	+
	100	–	–	–	+
	120	–	–	+	+
Ra	75	–	–	–	–
	100	–	–	–	–
	120	–	–	–	–

RAB = rabbit anti bovine antiserum
RASw = rabbit anti swine antiserum
RACh = rabbit anti chicken antiserum
RAKa = rabbit anti kangaroo antiserum

B = bovine antigen
Sw = swine antigen
H = horse antigen
O = ovine antigen
Ch = chicken antigen
T = turkey antigen
Os = ostrich antigen
Ka = kangaroo antigen
Ra = rat antigen

Twenty microlitres of antiserum and the same volume of the antigen (sample) were applied into the wells on the anode and the cathode sides, respectively. Protein migration proceeded under 4 W on one plate, i.e. 16 mA at 250 V for 45 min in the medium of Tris succinic acid. Bio-RAD Power Pac 3000 and TEP-2 (Sevac, Czech Republic) were used as the power supply and the electrophoretic units, respectively. Results were obtained after 48 hrs after washing the plates in PBS, wraping up Whatman paper No. 4, and staining of the plates with amidoblack 10B (Lachema, Czech Republic) following destaining at the destaining solution and parching at the laboratory temperature till next day.

RESULTS AND DISCUSSION

The best results in terms of specificity and sensitivity were obtained with the antisera RAB 75/2, RASw 100/5, RACh 100/2 and RAKa 100/7. The antiserum RASw 100/5 did not show any cross-reactivity and no absorption was necessary. The sensitivity of CIE for the detection of chicken, swine, beef and kangaroo proteins tested in dilution series of the immunisation antigens, was 1.5%, 5%, 5%, and 5%, respectively.

The specificity of the antisera expressed in terms of cross-reactivity with heterologous antigens is shown in Table 1. While the reactions of the antisera to porcine antigen were strictly species-specific, chicken antiserum yielded false positive reaction with the extract of kangaroo meat and processed at 120°C, bovine antiserum yielded false positive reaction with an exctract containing porcine antigen processed at 120°C, and kangaroo antiserum yielded false positive reactions with the extracts containing sheep antigens processed at 75 or 100°C and with the chicken, turkey and ostrich extracts processed at 120°C. The antisera were succesfully saturated by addition of cross reacting antigens in the amount corresponding to 10% of the total antiserum volume and subsequent centrifugation at 150 000 g and 4°C for 2 h. The only exception was bovine antiserum in which even repeated saturation procedures failed to eliminate reactions with porcine antigen heated to 120°C. Hence, the bovine antiserum is suitable for testing of meat products heated less than 100°C.

Fifty commercial heat-processed meat products (salamis, frankfurters, cooked ham, sausages, pork and beef in natural juice, canned ham, fresh meat, boiled pressed meat, canned sandwich spreads, and canned meat) were tested by CIE. In most cases, the test results were consistent with the declared composition but in seven cases adulteration was found. Chicken protein was demonstrated in five products declared as containing beef and/or pork only. One product contained undeclared bovine protein and another undeclared kangaroo protein. These positive samples were tested repeatedly ten times during ten following days with the same results.

In our experiments, the specificity of CIE with one unsaturated and three very low-saturated antisera was high enough to distinguish among phylogenetically distant species.

It can be concluded from the results of our experiments that CIE is an inexpensive, simple, and sensitive method for the identification of species-specific proteins in heat-processed meat products.

REFERENCES

Berger R.G., Mageau R.P., Schwab B., Johnston R.W. (1988): Detection of poultry and pork in cooked and canned meat foods by enzyme-linked immunosorbent assays. J. AOAC, *71,* 406–409.

Cutrufelli M.E., MageaU R.P., Schwab B., Johnston R.W. (1987): Detection of beef and poultry by serological field screening, (ORBIT and PROFIT): Colaborative Study. J. AOAC, *70*, 230–233.

Chin-Sheng Cheng Parish F.C. (1979): Heat-induced changes in myofibrilar proteins of bovine longissimus muscle. J. Food Sci., *44*, 22–24.

Hayden A.R. (1977): Detection of chicken flesh in beef sausages. J. Food Sci., *42*, 1189–1192.

Hayden A.R. (1979): Immunochemical detection of ovine, porcine and equine flesh in beef products with antisera to species myoglobin. J. Food Sci., *44,* 494–500.

Hayden A.R. (1981): Use of antisera to heat-stable antigens of adrenals for species identification in thoroughly cooked beef sausages. J. Food Sci., *46*, 1810–1813.

Hofmann K. (1977): In: Hoyem T., Kvale O. (eds.): Physical Chemical and Biological Changes in Foods Caused by Thermal Processing. Applied Science Publishers Ltd., London. 311 pp.

Hofmann K., Fisher K., Miller E., Kemper V. (1996): Experiments to demonstrate the effectiveness of heat treatments applied to canned meats and meat-and-bone meals. Fleischwirschaft, *76*, 920–923.

Kang'Ethe E.K., Gathuma J.M. (1987): Species identification of autoclaved meat samples using antisera to thermostable muscle antigens in an enzyme immunoassay. Meat Sci., *19*, 265–270.

Kang'Ethe E.K., Lindqvist K.J. (1987): Detection of beef and pork in fresh and heat-treated meat products using

enzyme immunoassay. J. Food Sci. Technol., *24*, 289–293.

Krcmar P., Rencova E. (2001): Identification of bovine-specific DNA in feedstuffs. J. Food Protect., *64*, 117–119.

Mageau R.P., Cutrufelli M.E., Schwab B., Johnston R.W. (1984): Development of an overnight rapid bovine identification test (ORBIT) for field use. J. AOAC, *67*, 949–954.

Martin R., Azcona J.I., Tormo J., Hernandez D.E. (1988): Detection of chicken meat in raw meat mixtures by a sandwich enzyme assay. Int. J. Food Sci. Technol., *23*, 303–310.

Martin R., Azcona J.I., Tormo J., Hernandez D.E. (1992): Immunoadsorption chromatography. Partial purification of pig-specific muscle soluble proteins. Fleischwirtschaft, *72*, 916–917.

Matsunaga T., Chikuni K., Tanabe R., Muroya S., Shibata K., Yamada J., Shinmura Y. (1999): A quick and simple method for the identification of meat products by PCR assay. Meat Sci., *51,* 143–148.

Milgrom F., Tuggac M., Witebsky E. (1963): Immunological studies on adrenal glands. III. Interspecies relations of thermostable adrenal-specific antigens. Immunology, *6*, 105–118.

Patterson R.L.S., Jones S.J. (1989): Species identification in heat processed meat products. In: Proc. 35th Congr. Meat Sci. Technol., Vol. II, 529–536.

Patterson R.M., Whittaker R.G. (1984): Improved species identification of raw meat by double sandwich enzyme-linked immunosorbent assay. J. Sci. Food Agric., *35*, 1018–1023.

Pickering K., Bazeley J., ClaY A. (1992): Meat speciation – a review of the specifity of commercial antisera. Lab. Practic., *41*, 59–60.

Reddy P.M., Reddy V.S.L., Rao Z.S., Murthy G.K. (2000): Identification of origin of fresh, cooked, and decomposed meats using brain antigens. J. Food Sci. Technol., *37*, 201–203.

Rencova E., Necidova L., Svoboda I. (2000): Identification by ELISA of poultry, horse, kangaroo, and rat muscle specific proteins in heat-processed products. Vet. Med. – Czech, *45*, (12): 353–356.

Sherikar A.T., Khot J.B., Jayarao B.M., Pillai S.R. (1988): Use of species-specific antisera to adrenal heat-stable antigens for the identification of raw and cooked meats by agar gel diffusion and counter immunoelectrophoretic techniques. J. Sci. Fd Agric., *44*, 63–73.

Sherikar M., Karkare V.D., Khot J.B., Jayarao B.M., Bhilegaonkar K.N. (1993): Studies on thermostable antigens, production of species-specific antiandrenal sera and comparison of immunological techniques in meat speciation. Meat Sci., *33*, 121–136.

Swart K.S., Wilks C.R. (1982): An immunodiffusion method for the identification of the species of origin of meat samples. Aust. Vet. J., *59*, 21–22.

Tremlova B. (2000): Histologischer Nachweis von Knochenpartikeln in Fleisch produkten. Fleiswirtschaft*, 80*, 73–74.

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