DETECTION OF SPECIFIC BOVINE PROTEINS IN HEAT-PROCESSED MEAT PRODUCT USING RABBIT ANTISERUM

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Abstract

The aim of the study was to obtain rabbit serum against proteins from heat-processed bovine muscle tissue. The immunogen was prepared by extracting a homogenate of beef muscle tissue and subsequent heat processing at 75, 100 and 121 °C. Electrophoretic comparison of the heat-processed and non-processed extracts revealed denaturation of almost all proteins except those with the molecular weight of 34–38 kD. After centrifugation, condensation and heat processing the extract was subjected to gel chromatography on a column with Sephadex G 75. For rabbit immunisation a fraction from the first elution peak was used. Only one precipitation line could be found when the quality control of antibody production as well as sample analysis were carried out by immunodiffusion precipitation on agarose gel. The using of the immune serum revealed the presence of bovine proteins. In model samples of mixtures (1 = beef and pork; 2 = beef and mutton and deer meat; 3 = beef and canine meat) beef was detected if present at concentrations of 5, 10 and 2%, respectively.

Key words: beef, heat processing, proteins, identification, immune serum.

Identification or authentication of the basic components of meat products has gained importance especially under the conditions of the EU-accession of this country. There are several reasons for the identification or detection of different kinds of meat, the main ones being related to economical, hygienic, as well as ethical viewpoints. In the period of intensive market mechanisms the economic viewpoint has been gaining importance. The necessity to solve the question of designing genuine tests for the detection of different meat species proteins has been indirectly emphasised (14). Ruggeberg and Gaade (15) obtained similar results of test authentication, however, with considerably higher financial inputs.

The Act of the National Council of the Slovak Republic No. 152/1995 of the Collection of Laws on Foods considers foods to be adulterated if their appearance, taste, composition or other characteristics had been changed by interference to an extent that decreased their nutritional value but which are offered to the consumer under their trade name. Protein detection may be of importance from the ethical and religious point of view.

The increased international meat trade is accompanied by efforts to adulterate meat products. For this reason it is important to check during distribution the identity of the animal species that had been the raw material source of the meat and meat products or that had been incorporated in the product (19). Of the other currently used methods the detection of adulteration of meat products using infrared microscopy (4) or quantitative estimation of pork adulteration can be mentioned. These rather reliable data were confirmed by Martin et al. (8) who used radial immune diffusion and the ELISA method. Ding and Xu (1) detected beef hamburger adulteration by the technique of close infra-electroscopy. Kangethon et al. (5) reported cases of adulteration of beef with frozen horse meat. With respect to manipulation and the free feed market BSE has gained importance and requires increased attention to be paid to reliable detection or identification of animal proteins of unwanted species (17). In adulterated food products the changes of quality usually occur due to intentional addition of certain substances in order to increase the volume or weight of the product, partial or total restriction of certain valuable components or replacement of the latter by constituents and raw materials of lower value. Suhaj and Kováč (18) and Pipek et al. (10) pointed out individual cases of meat replacement by another similar raw material.

The aim of present study was to prepare a method for using rabbit immune serum to detect bovine proteins in a set of meat product samples.

Material and Methods

Preparation of antigen. The bovine muscles were freed of fat by aceton at 4 °C for 24 h. The muscle were homogenised with 0.05 M PBS buffer (0.05 M
sodium phosphates + 0.15 M NaCl, pH 7.40) at a ratio of 1:1. The homogenate was extracted at 4°C for 12-18 h and divided into three identical weight parts which were then submitted to heat processing for 30 min at 75°C, 100°C and 121°C. Cooling was followed by centrifugation at 1000g for 20 min. The supernatant obtained was dialysis-condensed against 50% polyethylene glycol for 24 h. A 3 ml sample was taken from the condensed meat extract and mounted on a column (3.2 x 85 cm) with Sephadex G 75 which was equilibrated with 0.02 M PBS (0.02 M sodium phosphate + 0.15 M NaCl, pH 7.00). The column was eluted by the same buffer and the individual fractions were collected by a fraction collector.

The fractions belonging to the individual elution peaks were mixed and condensed by membrane ultrafiltration through a PM 10 membrane on an AMICON 8050 concentration unit. The molecular weight of the analysed proteins were compared with the set of standards and expressed as kD (Promega, USA).

Heat-processed homogenate of bovine muscles was used as antigen. The fractions of the first peak value (34-38 kD) were used for antiserum production.

**Preparation of antiserum.** Rabbits were immunised with 4 doses of immunogen by consequently increased concentration (30 µg, 50 µg, 150 µg). One ml of the antigen was mixed with 1 ml of Freund adjuvant. The mixture was thoroughly stirred until a thick, milk-coloured emulsion resulted which was then applied to rabbits of the New Zealand White breed. The injection was applied subcutaneously to 4 sites in the scapular region. Altogether 6 injections were given in 3-4 week intervals. Prior to the third and each following injection control blood samplings were carried out.

**Detection of meat protein.** The model samples of meat mixtures (5 g) were prepared in the following rate: (beef and pork 5:95; beef and mutton 10:90; beef and deer meat 10:90; beef and canine meat 2:98). The similar way of sample preparation was used in the following heat-processed commercial products of Slovakia origin: (President, Ščípak, Štart, Malokarpatská, Belianska, Kriváň and Tokaj salami, Bratislava and Súľov sausage) as well as of Austrian origin (Knoblauch stangerl).

Detection of meat protein was carried out by a modified immune precipitation method based on the method of Ouchterlony. The bottom of a 6 cm Petri dish was covered with 5 ml of 1% agarose solution cooked in 0.02 M PBS, pH 7.4. After agarose congelation and equilibration at 4°C for 30 min a template and a cork borer were used to drill 7 holes (1 central and 6 peripheral) in agarose. Rabbit immune serum was added to the central well with rabbit serum and the external wells No. 1, 2, 3, 4, 5, 6 were left to the peripheral wells. The reaction was incubated at 4°C for 12-14 h. The positively contact line was seen between antiserum and antigen concentrations 1:199 and 1:9, respectively. If this concentration was kept, the precipitation zones obtained were sufficiently sharp and did not diffuse. As a negative control there was always used the rabbit serum with bovine protein.

**Results**

In the present work, the lowest detection limit of bovine proteins were observed in used model mixture samples. The obtained results are recorded in Table 1. In these samples of mixtures (1 = beef and pork; 2 = beef and mutton and deer meat; 3 = beef and canine meat) beef was detected if present at concentrations of 5%, 10% and 2%, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Meat mixture</th>
<th>Immunoprecipitation</th>
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<tbody>
<tr>
<td>beef and pork</td>
<td>5%</td>
</tr>
<tr>
<td>beef and mutton</td>
<td>10%</td>
</tr>
<tr>
<td>beef and deer meat</td>
<td>10%</td>
</tr>
<tr>
<td>beef and canine meat</td>
<td>2%</td>
</tr>
</tbody>
</table>

Visual estimation revealed that after heating the meat samples over 60°C protein precipitation occurred in the form of a white precipitate. We observed most proteins with a molecular weight of more than 40 kD to be denatured.

Using gel chromatography with a Sephadex G 75 column non-denatured proteins of the 34–40 kD range were divided into three peak values. The third peak value contained only low-molecular fractions. The second and first peak value contained fractions with a relative molecular weight of 14 and 34-38 kD, respectively. The fractions of the first peak value (34-38 kD) were used for antiserum production.

The detection of different sources of raw materials is often combined with the problem of the technical reliability. In our study we observed positive immunoprecipitation on agarose between the central well with rabbit antiserum and well No. 6 with an antigen from bovine meat (Fig. 1).

It may be concluded that the modifications of immunogen preparation used in our experiment had positive effect on the production of rabbit antiserum in heat-processed samples. A positive immunoprecipitation reaction was stated in the following heat-processed commercial products: President, Ščípak, Štart, Malokarpatská, Belianska (Fig. 2), Kriváň and Tokaj (Fig. 3) salami as well as Bratislava and Súľov sausage (Fig. 4). These products contained mixture of beef and pork meat. On these products obtained from the supermarket a pronounced precipitation zone occurred. The positively contact line was seen after 12 – 14 h.

Similarly, positive immunoprecipitation reactions (Fig. 5) were observed between the agarose central well with rabbit serum and the external wells No.
4 and 5 that contained antigens obtained from *Knoblauch stangerl*, (mixture of beef and pork meat) a commercial heat-processed product of Austrian origin.

In the remaining external wells (No. 1, 2, 3) negative immunoprecipitation occurred indicating that the evaluated meat products did not contain bovine protein. This was a confirmation of the declared characteristics of the following meat products: country ham, cold turkey ham cut, ham mousse.

**Fig. 1.** Positive immunoprecipitation reaction on agarose with rabbit antiserum in the middle hole. The heat-treated antigen from beef from Slovakia is found in external hole No. 6.

**Fig. 2.** Positive effect on the production of rabbit antiserum in heat-processed samples (salami) from Slovakia.
The holes: No. 1 – President; No. 2 – Ščipak; No. 4 – Start; No. 5 – Malokarpatska; No. 6 – Belianska.

**Fig. 3.** Positive effect on the production of rabbit antiserum in heat-processed samples (salami) from Slovakia.
The holes: No. 1 – Kriváň; No. 2 – Tokaj.

**Fig. 4.** Positive effect on the production of rabbit antiserum in heat-processed samples (sausage) from Slovakia.
The holes: No. 1 – Bratislava; No. 3 – Súľov.
Discussion

From the hygienic point of view protein identification may be suggested in order to prevent substitution of meat intended for human consumption with unsuitable kinds of meat. In order to be able to check whether the declared properties of food products are really present, the inspection bodies of the State Veterinary and Food Administration need to differentiate the individual sorts of meat really incorporated in the marketed products. Determination of the individual sorts of meat and quantification of manipulation by other substitutes of animal or plant origin is an inevitable condition of quality of the distributed products. Test authentication is often confronted with the problem of high costs and the technical reliability detection of different sources of raw materials (14).

Falsely positive reactions occurring due to cross-reactions were not observed in the experiments. It is an important finding of our work that the method used for preparation of bovine proteins for rabbit immunisation yielded no cross reactions as in the case of animals phylogenetically related to each other. In previous experiments with antiserum obtained from serum proteins positive cross-reactions were regularly observed (16).

Similarly Reddy (12) observed cross-reactions in experiments using the DID, IE, CIE and RIE techniques. He focused on animals that were phylogenetically related to each other (buffalo, cattle, sheep, goat) and reported a 1% sensitivity of the method whereas in cases of heat-processed product analysis he even reported a sensitivity of 10-20%.

Manal et al. (7) identified the species origin of the raw material on the basis of animal hair and skin detected in the product. The morphological and histological method was aimed at animal species such as buffaloes, camels, donkeys and dogs.

On the basis of experimental work presented by Herich (1999, 2001) it can be presumed that in gnotobiotic piglets the immunity process and growth of immune competent cells leads to a strict specificity of IgG to the given animal species. Thus so the immunoprecipitation tests using meat substance of such animals to prepare rabbit antiserum against bovine proteins may be expected to ensure pronounced specificity to this antiserum. In such cases samples from phylogenetically related animals should not yield so-called cross-reactions in the immunoprecipitation test.

The fact that products containing separated meat (mechanically deboned) are rather difficult to detect remains to be a problem in the use of our antiserum. Confirmation of the presence of separated meat in the product is also based on the use of histological analysis of bone tissue by target staining (20).

Marcin et al. (9) introduced a method of plant protein (phytocomponent) detection in heat-processed meat products aimed at the detection of soybean proteins. The ELISA method (11) is similarly used in the dairy industry to detect dairy product adulteration. Lopez and Valencia (6) elaborated an SDS-PAGE method for the identification of animal proteins (milk, eggs) as well as plant proteins (rice, maize, wheat, soybean, mustard). Indirect competitive ELISA for specific muscle tissue identification of heat-processed poultry, horse, kangaroo and rat reached a sensitivity of 1-5% (14).

Undoubtedly the modifications of immunogen preparation employed in our experiment yielded the required positive effect supporting the production of rabbit antiserum that ensure immunoprecipitation also in heat-processed meat and meat product samples.

We suppose that it will be possible to use the proposed method in preparing immunogen from the meat of animals unsuitable for production and thus consumption (other animal species). A supply of antiserum will be established for detecting proteins of animals that got into the product (during the technological process) by chance or intentionally (for economic reasons). This enables to detect non-declared meat in the products.

In conclusion it may be stated that a method has been developed to prepare an immunogen for the production of specific rabbit antiserum against proteins from heat-processed beef that can be used to detect bovine proteins in meat products.

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References


