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APPLICATION OF THE "MYOGLOBIN METHOD" FOR THE IDENTIFICATION OF MEAT SPECIES IN HEATED MATERIALS

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SUMMARY

Isoelectric focusing (IEF) in rehydratable polyacrylamide gels and specific staining of myoglobins based on their pseudoperoxidase activity was tested for their aptitude to meat species identification. Materials under investigation were meat juices from cattle, sheep, horse, pig, rabbit, domestic rabbit, hare, deer, fallow deer, saiga, blackbuck, eland, blesbock, australian wild boar, chicken, turkey, duck, goose, wild duck, ostrich and extracts of raw and heated meats from cattle, sheep, horse, pig, rabbit, deer, chicken and turkey. However, only meat from animals which are not too closely related can be identified by this method.

As the visualization with the pseudoperoxidase staining of the myoglobins is very sensitive it allows the identification of meat species by the myoglobin method in the case of pale meat and in meat heated to 100°C. The intensity of the myoglobin bands is weakened by increasing heat treatment whereas their position is not influenced. In the case of most of the heated pale meats the extracts have to be concentrated by adsorption on hydroxylapatite so that the electrofocused myoglobins can be visualized. The electrophoretic and staining procedure is finished after about two hours.

The dried gels can be stored for documentation without change of the colour or loss of intensity of the stained myoglobin bands.

The specific visualization of the electrofocused myoglobins is a suitable method for meat species identification in meat with low myoglobin content and heated materials.

INTRODUCTION

Meat species identification is often carried out by electrophoretical methods especially by isoelectric focusing (IEF) because of its high resolving power. In most cases the separated proteins are visualized by a protein dye (Coomassie Blue) exhibiting complicated patterns. General problems of meat species identification using isoelectric focusing were discussed in several publications (HOFMANN, 1985; 1986a). First of all the protein patterns are variable depending on different influences. An improvement is obtained by the visualization of specific proteins (e.g. enzymes) (KING and KURTH, 1982; KING, 1984) or the bands of myoglobins separated by IEF which can be recognized by their own reddishbrown colour, the so-called myoglobin method (HOFMANN, 1986b; HOFMANN and BLÜCHEL, 1986). This method is well suitable for animals which are not closely related. However, the detection of meat species with low content of myoglobin or pale pieces of meat is connected with some difficulties. Furthermore heated meat can be identified only under certain conditions caused by the decreased solubility of heat-denaturated myoglobins.

The aim of our investigations was to increase the sensitivity of the myoglobin method so that it can be applied to pale and heated meats. A specific staining method for hemproteins (OWEN et al. 1958) seemed to be most promising in this respect. This method is based on the ability of the hemproteins to catalyze the oxidation of o-dianisidine by hydrogen peroxide (pseudoperoxidase activity) forming an intense brown colour marking the myoglobins in the gel. This principle has already been used to investigate the heterogenity of the myoglobins in PSE and normal muscles of pig (WYKLE et al., 1978). In addition the usefulness of rehydratable polyacrylamide gels had to be checked for the investigation of meat and meat products because salts which occur in press juices and extracts of meat and meat products can cause trouble in the separation of the proteins (wavy bands). Rehydratable gels offer the possibility of investigating meat samples with unavoidable high salt concentrations (FREY et al., 1986).

MATERIALS AND METHODS

Materials under investigation

Meat juices from cattle, sheep, horse, pig, rabbit, domestic rabbit, hare, deer, fallow deer, saiga, blackbuck, eland, blesbock, australian wild boar, chicken, turkey, duck, goose, wild duck, ostrich and extracts of raw and heated meat from cattle, sheep, horse, pig, deer, rabbit, chicken and turkey were used. Homogenized meat samples were heated in vacuum bags under variable conditions (until 100°C, details see "results and discussion"). In order to obtain reproducible and comparable results in heated samples the minced meat in the bags was pressed smoothly to a 3 mm thin layer.

Sample preparation

Press juicesare prepared by squeezing meat samples between two glass plates. The meat juices are then diluted by adding a solution of 0.001 M $K_3[Fe(CN)_6]$, filtered through a folded filter and if the filtrates are turbid clarified by a cellulose acetate filter (0.2 µm pores). The extent of dilution of the press juices depends on the species; juice and $K_3[Fe(CN)_6]$-solution being mixed as follows : poultry, rabbit and other pale meats 1+1 to 1+2, beef, sheep, pork and wild poultry 1+4 to 1+10, game and horse 1+10 to 1+20. Minced meat is extracted with a solution of 0.001 M $K_3[Fe(CN)_6]$ for 15 min under occasional stirring followed by filtration and clarification as described above.

Heated meats are extracted by suspending 1 part of minced meat in two parts of a 0.075 M Tris/HCl buffer pH 8.9 using a homogenizer (Ultra Turrax). This suspension is incubated for 3 hours to 37°C followed by centrifugation (g_{max} at least 1500g). The supernatant fluid is filtered and clarified as described above.

Gel preparation

0.5 mm thick polyacrylamide gels (5% total acrylamide T, 3% crosslinker C; gel dimension 125x265 mm) containing 0.375 M Tris HCl buffer pH 8.9 are prepared on polyester supports (Gel-Fix from Serva) using the capillary technique (SERVA product information, Merkblatt Nr. 116, catalogue 1986/87). The gels are washed twice for at least 30 min with deionized water and than placed in a solution of 3% sorbitol (w/v) and 2% polyvinylpyrrolidone (M.W. approx. 10000) (w/v) for at least 30 min. The gels are dried at room temperature.

The dried gels are rehydrated by the mould technique (GÖRG et al., 1979). The solution for rehydration contains 3% carrier ampholytes (w/v) and

10% glycerol (v/v) in water. The time for rehydration is at least 3 hours.

Concentration of the extracts

20 ml of the extract are applied to a chromatographic column with a small diameter (e.g. 1 cm internal diameter) containing 1 ml hydroxylapatite. After adsorption the proteins are desorbed with a 0.2 M phosphate buffer pH 7. The eluant is collected in 0.5 ml fractions. The fraction with the most intense reddish-brown colour is used for the IEF. Alternatively the concentration can also be carried out by batch adsorption on hydroxyl apatite. 0.5 g dry powder of hydroxylapatite is added to 10 ml of the extract in a centrifugation tube. The adsorption is carried out by occasional stirring for 15 min. After centrifugation and throwing away the supernant fluid the proteins are desorbed with 0.5 ml 0.3 M phosphate buffer pH 7 by stirring and centrifugation. The supernant fluid is used for IEF.

Electrophoretic procedure

Pre-electrophoresis is carried out at a constant power at 40 V/cm in the beginning. The distance of the electrodes are approximately 10 cm. After the voltage has reached 80 V/cm the IEF is interrupted and the samples (10 µl each) are applied to the gel using a silicon applicator strip put on the gel in a distance of 2-3 cm from the anode. Afterwards the voltage is set up to 400 V for 10 min. Then the electrophoresis is carried out at 20 W constant power and a final voltage of 1700 V. The IEF is finished when the myoglobin standard applied at the anodic and cathodic site have reached the same position (i.e. the isoelectric point) in the gel.

Staining procedure

0.1 g o-dianisidine is disolved in 30 ml ethanol followed by the addition of 70 ml 0.1 M citrate/ 0.2M phosphate buffer pH 5 and directly before use 2 ml hydrogen peroxide. Within 15 min the formation of an intense brown-red colour is finished. Further details are given by BAUER and HOFMANN (1987).

RESULTS AND DISCUSSION

Raw meat

Most of the slaughter animals, game and domestic and wild poultry investigated, can be identified by this method in which a pH-gradient from 3 to 10 is used. The electrophoretic procedure is finished after about 120 min. A typical pherogramme is shown in Fig. 1. Closely related animals e.g. different species of antilopes, chicken and turkey or duck and goose as well cannot be distinguished in this way. Even if gels with a narrow range pH-gradient (e.g. 6-9 or 5-8) are used in which the bands are separated further the meat species mentioned above cannot be identified.

However, the identification of all species is easier in the latter case because the distances between the myoglobin bands are enhanced. On the other hand the electrophoresis under these conditions takes more time in comparison to the gel pH 3-10.

With exception of the extracts of chicken meat (see Fig. 3) all myoglobins of meat species under investigation could be visualized by the described staining method.

In contrast to the whole protein patterns of the meat extracts the myoglobin patterns are not influenced during storage. For example storage at 4°C for several days had no influence on the myoglobin patterns but on several sarcoplasmic proteins which were precipitated in the extract. A solution of $K_3[Fe(CN)_6]$ was used for diluting the press juices for the extraction of minced meat in order to obtain an uniform state of oxidation (different contents of oxi- and metmyoglobin in meat samples may cause variations in the myoglobin patterns).

Heated meat

It was found that the myoglobins are less sensitive against heat than most of the other sarcoplasmic proteins. Therefore the "myoglobin method" is very useful for the detection of meat species in heated samples.

The investigation of the influence of the heat treatment on the myoglobin bands studied on samples from beef showed that they can still be recognized by their own reddish-brown colour, if the samples were heated not longer than 60 min to 70°C or 30 min to 75°C. Using the pseudoperoxidase staining the myoglobins can still be visualized in samples heated 60 min to 100°C. The intensity of the myoglobin bands decreases whereas their positions are not influenced by the heat treatment (Fig. 2).

During the extraction of heated meat samples addition of $K_3[Fe(CN)_6]$ is not necessary because the iron in the myoglobins denaturated by heat treatment exist in the trivalent state. The extracts of chicken, turkey, rabbit and pig were turbid and could not be clarified by membran filtration. However this did not influence the results of the IEF.

In Fig. 3a IEF pherogramme of different raw and heated meat species is presented. The increase of the protein concentration of the extracts of heated meats was achieved by batchwise adsorption on hydroxylapatite. With expection of pale pork (loin) and chicken breast muscle all meat species under investigation could be visualized. Pale meat heated to 100°C has to be concentrated using the column procedure. But again chicken breast muscle and pale pork could not be visualized by this method.

With respect to the meat species identification of meat products we found, that salting and curing of meat doesn't influence the position of the myoglobin patterns and the pseudoperoxidase staining as well.

Additional experimental remarks

IEF in rehydratable polyacrylamide gels was especially advantageous for meat species identification since this type of gels is less sensitive to salt in the sample than the gels usually prepared. Therefore meat juices and meat extracts as well do not need to be desalted even if the extracts contain relatively high amounts of salts (e.g. phosphate buffer used for protein desorption from hydroxylapatite or sodium chloride in extracts of meat products).

The most sensitive substrate under investigation was o-dianisidine as described by OWEN et al.(1958). But another buffer had to be used in order to achieve a suitable sensitivity. o-Toluidine which is recommended by KINZKOFER and RADOLA (1983) has proved to be not sensitive enough for the visualization of myoglobins. Other substrates like o-tolidine, 3,3'-diaminobenzidine and 3-amino-9-ethylcarbazol did not prove suitable either for this purpose in our experiments.

The stained gels can be dried easily and stored at room temperature for a long time as a record without changing the colour of the myoglobin patterns and their intensity.

CONCLUCIONS

(1) Meat species can be identified in general by the myoglobin patterns also in heated meat samples.

(2) The application of the "myoglobin method" to heated meat is possible by using the pseudo-peroxidase staining wich increases the sensitivity of the detection considerably.

(3) The investigation of samples containing salt can be carried out without desalting the extracts using rehydratable polyacrylamide gels.

(4) In principle the method is also applicable on salted and cured meat products.

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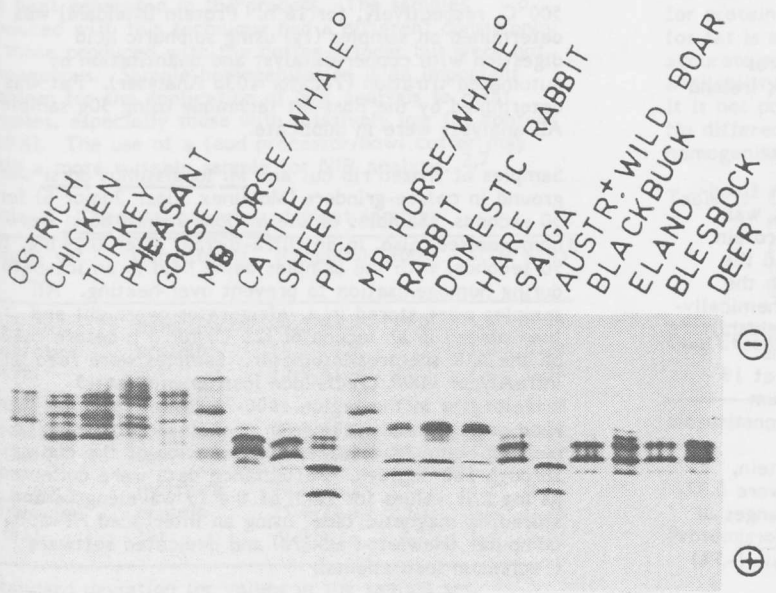
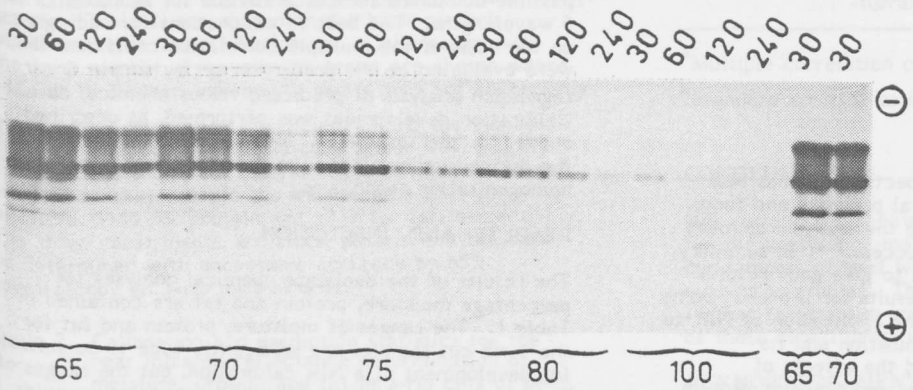


Fig. 1.
Isoelectric focusing of press juices from different animals; pH-gradient 3 - 10; pseudoperoxidase staining.
o = mixture of horse and whale myoglobin
+ = australian

— TIME IN MINUTES —



— TEMPERATURE IN °C —

$+\overline{K_3[Fe(CN)_6]}$

Fig. 2
Isoelectric focusing of extracts from beef heated under different conditions;
pH-gradient 5-8; pseudoperoxidase staining;
+ $K_3[Fe(CN)_6]$: addition of $K_3[Fe(CN)_6]$ to the extracts

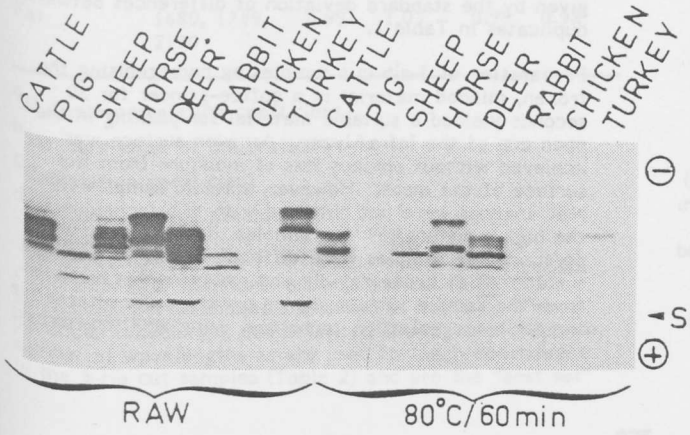


Fig. 3.
Isoelectric focusing of extracts of raw and heated meat from different animals;
pH-gradient 3 - 10; pseudoperoxidase staining.
S: site of sample application