Species Identification of Heated Meat and Meat Products by Isoelectric Focusing in Polyacrylamide Gels Followed by a Sensitive Silver Staining Method

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SUMMARY

Isoelectric focusing (IEF) of proteins of heated meat and meat products extracted with 0.075 M tris buffer, pH 8.9, using polyacrylamide gels enables the identification of beef, pig, horse, sheep, deer and several other meat species after visualization of the protein patterns in the IEF gel by a very sensitive silver staining method.

The samples investigated were canned products heated to different levels of stability (F_C-values: 0.2, 1.0, 4 - 6 and 12). A corresponding cooperative trial in which 22 institutes took part demonstrated the reliability of the method. Polyacrylamide gels of different thickness (0.15 - 0.5 mm) and different pH gradients (6 - 9; 3.5 - 9.5; 3 - 10) were used by the participants successfully. Investigations about meat mixtures of different species are in progress.

INTRODUCTION

Species identification of meat and meat products is an analytical task of increasing and worldwide importance for food inspection and control. Isoelectric focusing (IEF) of water-soluble muscle proteins in pH gradient gels is described as being very suitable for this purpose, and fundamental problems in identifying the animal species of muscle meat using electrophoretic methods have been discussed (HOFMANN, 1987). It was shown that the protein patterns of the total sarcoplasmic proteins in the gel may be influenced by a number of factors invivo but not the myoglobin bands, which are therefore the most suitable bands for identifying the animal species of muscle meat. In the case of raw meat the highly concentrated juices pressed or dripped can be applied and the species-specific myoglobin patterns are visible after IEF without staining procedure (HOFMANN and BLÜCHEL, 1986).

The sensitivity of this determination, however, can be greatly improved by peroxidase staining of myoglobins (BAUER and HOFMANN, 1987a, 1987b). This enables to use this method to samples, which are heated moderately up to 80°C or, in some cases to 100°C. The intensity of the myoglobin bands in the electrophoretic gel decreases with increasing temperature and heating-time (BAUER and HOFMANN, 1990a, 1990b).

The most sensitive and for heated meats most suitable method of visualization of proteins in the gel is the silver staining method. Meats from cattle, pig, sheep, horse, deer, rabbit, chicken and turkey and also frankfurter-type sausages made from the meat of these species and meat mixtures made from beef and pork or beef and mutton - all heated to 100°C - could be identified in all cases by means of this method (BAUER and HOFMANN, 1987b, 1989). The aim of our investigations was to check whether the silver staining method can also be extended successfully on meat heated to higher temperatures used for the production of canned meat products.

MATERIALS and METHODS

Outfit and Procedure for Isoelectric Focusing (IEF)

For IEF the "LKB Multiphor System" (Multiphor II Electrophoresis Unit, Macrodrive 5 Power Supply, Multitemp, mould for preparing gels) was applied. Polyacrylamide gels with different pH gradients (mainly pH 6 - 9) were prepared using Servalyt^R carrier ampholytes. Freshly prepared as well as rehydrated gels (after FREY et al., 1986; BAUER and HOFMANN, 1987) proved to be suitable. The principle of the IEF procedure used was described by GÖRG et al. (1979) and in the instructions of LKB. During IEF the temperature was kept at +8°C and the Power Supply was adjusted to 1700 V, 50 mA and 25 W. Under this conditions the focusing was finished after 2 1/2 hours.

Materials under Investigation

Muscular tissue of five different species - beef, pork, horse, sheep and deer - was investigated. Samples of lean meat were minced, partly mixed with 2 percent salt for curing (containing 99.5% NaCl and 0.5% $NaNO_2$), filled in cans (100 g each) and autoclaved under computer-controlled conditions. Four groups of products with different effects of sterilization were prepared, the F_C-values being 0.2 (pasteurized), 1 (3/4-conserved), 4 (fully conserved) and 12 (tropical conserves) respectively. The F_C-value is a measure for the intensity of heat treatment in the centre of the canned product. For example $F_C = 1$ means the product was heated to an extent which corresponds to an heating effect of 1 min to 121°C ($F_C = 4$ corresponds to 4 min at 121°C etc.).

Sample preparation for IEF

Heated meats were extracted by suspending 1 part of minced meat in 2 parts of a 0.075 M Tris/HCl buffer pH 8.9 using a homogenizer (Ultra-Turrax). This suspension was incubated for 3 hours to 37°C followed by centrifugation (at least with 1500 g). Finally the supernatant fluid was filtered in order to seperate traces of fat possibly occuring. The extracts (10 µl each) were applied directly to the gel with help of a silicon applicator strip put on the gel in a distance of 2 - 3 cm from the anode.

In some cases it was necessary to dilute the meat extracts described above (1 + 2 to 1 + 4). In other cases (e.g. chicken meat) it can be advantageous to concentrate the extracts. This depends on the intensity of heat treatment and of the original content of myoglobin in the meat sample investigated and has to be learnt by running a test gel.

Silver Staining and Preservation of the Gel

The success of investigation depends widely from the method of silver staining and is therefore given in detail (after HEUKESHOVEN and DERNICK, 1986, modified by BAUER and HOFMANN, 1987b). The different steps of gel treatment, carried out in a tray, are as follows:

1. After finishing the electrophoresis the gel is inserted to 10% trichloro acetic acid (fixing the proteins).
2. Incubation in a mixture of 30% ethanol, 0.5 M sodium acetate, 0.5% glutardialdehyde and 0.2% sodium thiosulfate.
3. Washing three times with water (400 ml each).
4. Gel treatment with silver nitrate solution containing 0.1% $AgNO_3$ for 0.3 - 0.5 mm gels and 0.02% formaldehyde (for 0.15 mm gels 0.3% $AgNO_3$ is recommended).
5. Development in a solution of pH 11.3 - 11.8 containing 0.01% formaldehyde and 2.5% sodium carbonate.
6. The staining procedure is stopped after removing the developing solution by adding 250 ml of water; we found that the additon of EDTA as recommended by HEUKESHOVEN and DERNICK (1986) had no effect. Furthermore it was found, that sodium thiosulfate may be omitted in the incubating solution used in step 2.

After each step the gel is briefly rinsed with dist. water. The time needed for the different steps is listed in table 1. Further investigations have shown that the different intervals can be shortened considerably without disadvantage for the result (see table 1). In this way the time needed for the entire silver staining procedure could be reduced from originally several hours to 1 hour for thin (0.3 - 0.15 mm) gels and 1 1/2 hours for 0.5 mm gels (see table 1).

For conservation the gel is treated (15 min) with a preserving solution containing 50 ml 87% glycerin and 12.5 ml glacial acetic acid in 2500 ml and air-dried over night. We found that the water in step 6 can be replaced by the preserving solution thus simplifying the method. The gels can be preserved and stored unlimited periods by covering them with a gelatine coating (HOFMANN, 1991): The dry gel is immersed in an aqueous 10% solution of highly viscous gelatine (e.g. edible gelatine, 280 Bloom grade) at 55 - 60°C and dried by hanging it in air.

Table 1: Time intervals for the silverstaining procedure

	HEUKESHOVEN & DERNICK, 1986	BAUER & HOFMANN, 1987	HOFMANN, 1990	
	0.1 - 0.2 mm	0.5 mm	0.5 mm	0.15 - 0.3 mm gels
1. TCA treatment	0.5 - 3 h	0.5 h	30 min	20 min
2. Incubation	0.5 - 2 h	2 h	15 min	10 min
3. Washing	3 x 10 - 30 min	3 x 10	2 x 15 min	2 x 10 min
4. Silver solution	15 - 60 min	0.5 h	15 min	10 min
5. Development	5 - 15 min	10 min max.	few minutes	
6. Stopping	5 - 10 min	10 min	few seconds	

RESULTS and DISCUSSION

In fig. 1 and 2 typical IEF-gels after silver staining are shown presenting the protein patterns of the five meat species of heated products. Only the bands in the upper part (the basic range) of the gels can be regarded as characteristic and typical for the animals species. In contrast to raw meat (not presented in the figures) the heated meat samples show only few bands and therefore rather simple patterns. They correspond mainly to the patterns of myoglobin as already demonstrated (HOFMANN, 1989). The myoglobins are obviously somewhat more soluble after heat treatment than all other sarcoplasmic proteins.

Samples heated to the lowest extend (F_C = 0.2) show the clearest patterns (fig. 1). With increasing intensity of heating (rising F_C-values) the stained bands are becoming weaker and less distinct (fig. 2).

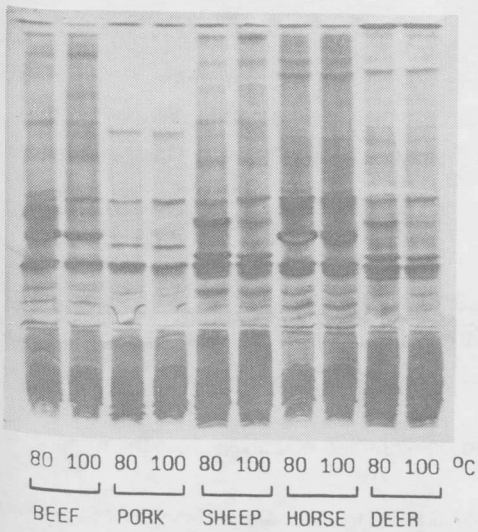

Fig. 1

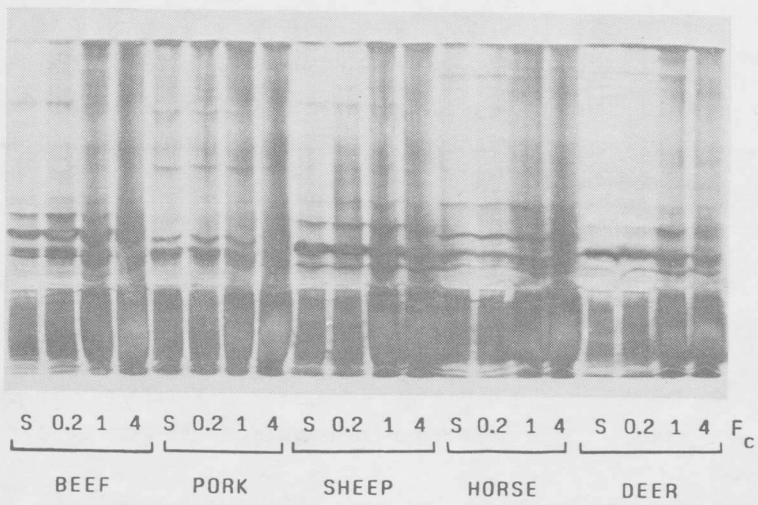

Fig. 2

Fig. 1: Silver stained protein patterns of beef, pork, sheep, horse and deer meat after heating to 80°C and 100°C, 30 min each (heating for 30 min to 100°C corresponds to F_C value of 0.2)

Fig. 2: Silver stained protein patterns of different meats heated to different extent of sterilization (definition of F_C value see text; S: standard sample, F_C = 0.2)

Simultaneously the background of the runs is getting darker and the clearness of the stained protein patterns decreases as a result of the increasing heating effect. Some bands even seem to disappear. Obviously not only denaturation of the meat proteins takes place during heating but also a decomposition, especially in the sterilized samples.

In all cases even with tropical conserves, however, the different meat species could be identified. Thus IEF and the modified silver staining method of HEUKESHOFEN and DERNICK (1986) allows an uncomplicated and safe identification of pasteurized or preserved meat of cattle, pig, horse, sheep, deer and probably of some more species which are different in their myoglobin patterns as shown by BAUER and HOFMANN (1987a). However, animals of different breed, race, sex or age cannot be distinguished by this method.

Further investigations have shown that the presence of salt in the meat samples do not have a negative effect on the result of IEF so that dialysis of the extracts is not necessary before their application to the gel.

In order to check the reliability and usefulness of this method of meat species identification for the application in meat inspection a cooperative trial was carried out in which 22 German official institutes participated. Codified meat samples of the same species and heated in the same way as the samples investigated in this study (without tropical conserves) were posted to the participants and analyzed. In all cases the samples were identified correctly. The method was therefore drawn up to the German collection of the official investigation methods. In addition the trial showed that gels of different thickness (0.15 - 0.5) and with different pH gradients (6 - 9, 3.5 - 9.5, 3 - 10) are in principle applicable. This method should also be applicable to meat mixtures of different species. The detection of small proportions of pork in heated beef/pork mixtures has already been described (BAUER and HOFMANN, 1987b). However, general investigations in this field were not carried out and are in progress.

The mechanism of reaction during silver staining may be as follows: First free silver ions (Ag^+) are bound unspecifically to the protein forming a protein-silver complex (1). Although the entire gel is impregnated by silver nitrate only the silver ions within the protein complex are reduced to metallic silver (Ag) by formaldehyde (2) added afterwards in basic environment:

$$(1) \quad x\,Ag^{+} + \text{Protein} \longrightarrow [\,Ag^{+}_{x} - \text{Protein}\,]^{x+}$$

$$(2) \quad [\,Ag^{+}_{x} - \text{Protein}\,]^{x+} \xrightarrow[\text{pH } 11.5]{CH_2O} x\,Ag \;\ldots\; \text{Protein}$$

The process is not influenced by light and is therefore not related to the photographic process. For interrupting the development only a decrease of the pH value (replacement of the developer by water or acid) is required, but not EDTA, which is not able to bind Ag^{+}-ions as assumed by HEUKESHOVEN and DERNICK (1986). On the basis of this knowledge the staining procedure could be simplified.

CONCLUSIONS

1. Isoelectric focusing (IEF) of heated meats extracted by tris buffer, pH 8.9, followed by a sensitive silver staining method, enables - in comparison with appropriate standard samples - the identification of meat species like cattle, pig, sheep, deer, horse and probably others being different in their patterns.
2. The IEF patterns of heated meats correspond mainly to the myoglobin bands. They are simpler than those of the unheated ones, because only the most resistent proteins (myoglobins) are able to be extracted effectively from the material denatured by heat.
3. For meat species only the bands in the basic range of the gel can be considered to be characteristic. However, the acid range gives also valuable informations about the intensity of heat treatment of the samples under investigation.
4. The distinctness of the species specific patterns decreases with increasing extent of heat treatment. Nevertheless in all cases the meat species of the different preserves could be identified.
5. Commercially available as well as self-made polyacrylamide gels with different thickness and pH gradients (preferably pH 6 - 9) may be used in this method.
6. The salt content in the products investigated did not disturb the result of IEF. However, rehydrated gels are even more salt tolerant than normal gels and therefore should be prefered.
7. The mechanism of silver staining is not yet fully understood. An explanation is given in accordance with our experiences.

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