IMMUNOLOCALISATION OF MYOFIBRILLAR PROTEINS IN DRY SAUSAGES: SELECTION OF SUITABLE ANTISERA AGAINST ACTIN AND MYOSIN.

Bart J. van 't Hooft *, Hein P.M. van Laarhoven **, Gerrit Wijngaards **, Joost Bruyntjes **.
*Stegeman , P.O.Box 93,Deventer, The Netherlands; **TNO Nutrition and Food Research Institute, P.O. Box 360, Zeist, The Netherlands

Introduction

Myofibrillar proteins, especially myosin and actin are important proteins in the formation of the structure of dry sausages (Kotter and Prändl 1958, Katsaras and Peetz 1988). The process of binding takes place in 3 phases: activation, diffusion and stabilisation. During activation by salt, added during bowlchopping, part of the protein solubilises. In the second phase these proteins diffuse from the myofibrils to form a matrix of protein and water outside the muscle cells binding together meat, fat and connective tissue particles. In the third phase, during fermentation, pH drops below 5.6 and a gelation takes places thus stabilising the system.

In order to understand the process of binding immunohistochemical techniques can be useful to identify and localise specific proteins in microscopic sections of meat, meat systems and meat products. Information about the distribution of functional proteins enables us to correlate microstructural phenomena with process parameters and functional properties of meat products.

Previously authors reported on difficulties with reactivity and non-specific staining of antisera. Zijderveld and Koolmees (1990) investigated the use of immunohistochemical techniques in order to identify specific muscle proteins in frozen and paraffin embedded sections of comminuted meat products. They found that the fixation procedure and the state of denaturation due to the presence of salt and heating of the specific proteins greatly influenced the reactivity of the antibodies.

Egelandsdal *et al.* (1991) tested 6 different antisera in an attempt to visualize changes during manufacturing of comminuted meat systems. They found 1. non-specific staining of other proteins than the antigen used for the production of the antiserum and 2. heat treatment reduced the reactivity of antibodies, but reactions were sufficiently strong to provide information about location of specific proteins.

Immunohistochemical techniques are possibly applicable in unheated sysems like dry sausages, where the denaturation is less severe and the binding places (epitopes) are probably partially lost. Investigation of the specificity and reactivity in several stages of dry sausage preparation is necessary to screen the usefulness of antisera.

Specificity of an antiserum mainly depends on the purity of the antigen used for the production of the antiserum. It can be estimated by Western Blotting after gel electrophoresis of protein extracts of meat samples. Reactivity is complex; the results can be influenced by the denaturation status of the protein and also by the extraction procedure and reaction conditions of Western Blotting.

In this paper methods and results of screening of 7 commercially available antisera for use in immunohistochemical localisation in dry sausages are presented.

Materials en Methods

Antisera: Three commercially available anti-actin antisera (coded A1, A2 and A3) and 4 commercially available anti-myosin antisera (coded M1, M2, M3 and M4) were used.

Secondary antibodies: For antisera developed in mice a horse radish Peroxidase conjugated Goat Anti Mouse (Immunoglobulin M, Fc) antiserum was used for Western Blotting and a Biotin labeled Rabbit Anti Mouse (Ig G) antiserum for immunostaining. For antisera developed in rabbit a Peroxidase conjugated Goat Anti Rabbit(Ig G, H+L) was used for Western Blotting and a Biotin labeled Pig Anti Rabbit (Ig G) antiserum for immunostaining.

Samples: Investigations on reactivity and specificity of the antisera were carried out on 2 groups of samples. Antisera A1, M1 and M2 were tested on freshly frozen beef and freshly frozen pork meat both from lots of shoulder meat for industrial processing.

Antisera A2, A3, M3 and M4 were tested on pork meat (*M. supraspinatus*) and beef (*M. semitendinosus*), which were frozen 24 hrs after slaughtering. All antisera were tested on freshly prepared dry sausage batter, frozen just after stuffing in casings and on finished product, after two weeks of fermentation and drying (losing about 22-25 % of the initial weight).

Immunolocalisation was carried out on postrigor pork meat and beef with antisera A1, M1 and M2 and prerigor pork meat (*M. supraspinatus*) and beef (*M.semitendinosus*) with the other antisera and on the same samples of dry sausage batter and finished product as in the Western Blotting experiments. Samples were fixed in formalin (1:10) immediately after sampling for at least 24 hours at room temperature for paraffin embedded sections and frozen after sampling for cryosections.

SDS-PAGE: SDS-PAGE was performed under unreduced and reduced conditions, using a modification of the method of Laemmli (1970). Extraction media were: Unreduced: 0.063 M Tris (hydroxymethyl) amino-ethane, 2 % SDS, 20 % glycerol, 0.01 % bromophenol blue in water (pH 6.8); Reduced: as unreduced, with addition of 1 % DTT (Dithyotreitol).

Samples and molecule marker (MW 14,000 - 200,000 Da) were put on 15 % Mini Protein II Tris HCl ready gels (Biorad). The electrophoresis buffer contained 0.3 % Tris-base, 1.44 % glycine and 0.1 % SDS. After electrophoresis the gels were stained with Coomassie Brilliant Blue (0.1 %) in 25 % methanol and 8 % acetic acid.

Western Blotting: Western Blotting was carried out according to Towbin et al (1979) using a PVDF (Polyvinylidinedifluoride) membrane (Immobilon-P, Milipore). After electrophoresis and washing in 0.1 % Tween-20 and 1 % skimmed milk (Protifar) in TBS (Tris base solution; 50 mM Tris,150 mM NaCl, pH 7.4) the PVDF membrane was incubated 4 hours with the test antiserum, washed again and further incubated with the peroxidase conjugated second antibody. Staining was carried out with Diaminobenzene Chloronaphtol reagent (44 ml TBS, 4 ml 0.3 % Chloronaphtol, 0.6 ml 3.3 % DAB and 20 ml 30% H_2O_2). Controls were made by the same procedure, omitting the first antibody reaction. The blots were digitalised with a scanner.

Immunolocalisation: Reactions with antisera were carried out both on paraffin embedded and cryosections.

Sections were rinsed with goat serum to reduce a-specific staining. Then the sections were incubated with the test antiserum, rinsed in PBS (Phosphate buffered saline; 0.1 M, pH 7.4) incubated with the biotin labeled secondary antibody, rinsed in PBS and then incubated with peroxidase conjugated streptavidin. Staining reaction was with DAB (5 mg DAB in 10 ml PBS with 5 µl 30 % H_2O_2). Sections were also

treated with Haemaluin for background staining. Controls were made by the same procedure, omitting the first antibody reaction step.

Results and Discussion

Reactivity and specificity: The best immunoreactivity was achieved under non-reducing conditions rather than under reducing conditions. Reduction probably decreased the number of possible epitopes on the protein molecules.

Western blotting after SDS-PAGE of the unreduced extracts of pork meat, beef, dry sausage batter and finished product indicated the specificity and the reactivity of the antisera for the use of immunostaining. Controls gave no significant reactions. Beside bands at 200 kDa (considered to be corresponding with myosin heavy chain) or at 45 kDa (considered to be corresponding with actin) several of the tested antisera revealed non-specific bands as can be seen in Fig. 1A. Correct binding with the actin and myosin proteins was considered because in sections of fresh material a regular pattern of striating (A-and M-)bands could be seen (Fig. 2A). Fig. 1B gives an example of a highly specific antiserum (M2), but this antiserum gave a very poor reaction with pork myosin (lane 2). Fig. 1C is a blot with a highly specific reacting anti-actin A2 (which appeared to be identical with A3), but no reaction took place with the finished product (lane 4). The anti-actin antiserum A1 was not specific enough to be used for immunolocalisation and also anti-myosin M1, M3 and M4 were not specific enough. Actually, antiserum M4 non-specific reaction with actin was higher than the specific reaction with myosin (results not shown).

Immunolocalisation: Typical colouring of fresh meat (beef) with anti-actin A1 can be seen in fig. 2A. A clear staining of striatings in the muscle cells could be observed in the fresh meat sections. Controls gave no colouring. Anti-actin A2 and A3 gave similar results and showed no non-specific staining. In the dry sausage batter the reactivity of anti-actin A1 with matrix-material was good, but poor with the intact myofibrils. Striatings in the muscles cells could still be observed. No reaction took place with the fat and cooked rind particles. The loss of reactivity with the antiserum may be due to the salt induced modifications.

Antiserum M1 reactivity with fresh meat was similar to the anti-actine antisera but non-specific colouring of the epimysium and perimysium could be observed (results not shown). In dry sausage batter reactivity with intact myofibrils was even poorer than of antiserum A1. Also non-specific staining of fat cell walls (F) and connective tissue (CT in Fig. 2B) was found. Antisera M2 and M3 gave similar results. A number of muscle cells in fresh meat were not stained at all with antiserum M4. This could be related to the type of fibers.

Cryosections gave a good reaction with antisera, but the lack of fixation resulted in the loss of the striating in the myofibrils. Therefore, a protein fixation with formalin prior to immunostaining seems necessary.

Conclusions

Of seven antisera tested only two identical ones (A2 and A3) were specific with good reactivity to be used for immunolocalisation in dry sausage batter.

Specificity and reactivity vary between the antisera and in different stages of processing the reactivity is influenced, probably due to the loss of epitopes.

For immunolocalisation purposes an investigation of the specificity and reactivity of the antisera is essential.

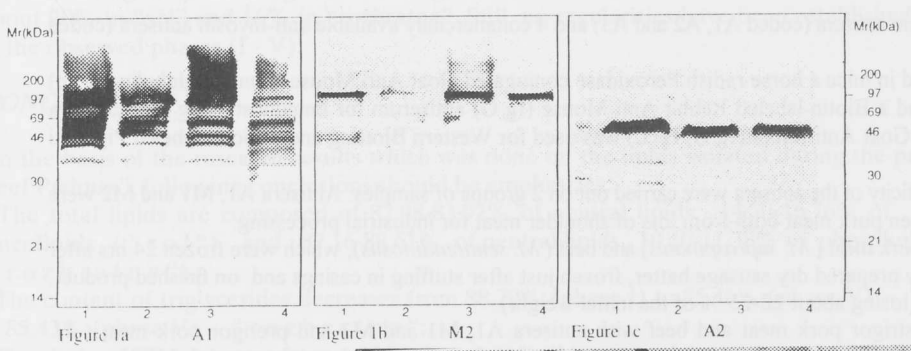

Figure 1a A1 Figure 1b M2 Figure 1c A2

Figure 1
Western blotting analysis under non-reduced conditions of protein extracts.
Figures 1a using anti-actin A1; 1b using anti-myosin M2;
1c using anti-actin A2.
Lanes 1. beef meat; 2. pork meat;
3. dry sausage batter; 4. finished product

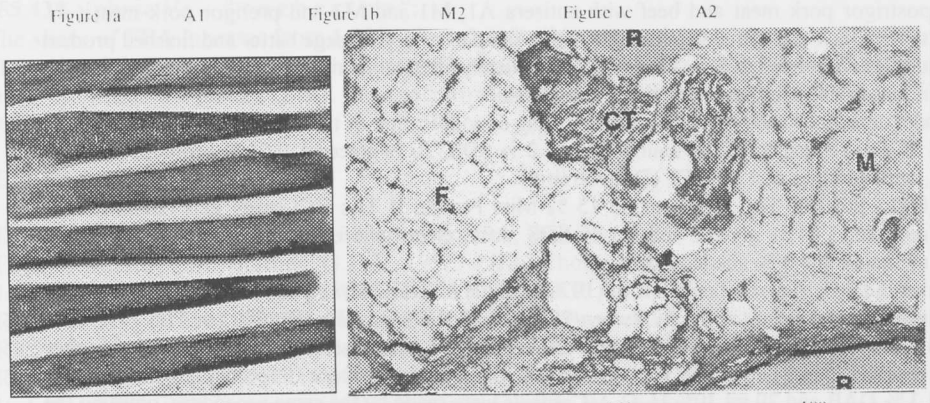

Figure 2a A1 400x Figure 2b M1 100x

Figure 2
Paraffin embedded sections of 2A: fresh beef. First Ab anti-actin A1;
2B: dry sausage batter.
First Ab anti-myosin M1.
M = Muscle tissue.
F = Fat tissue.
CT = Connective tissue.
R = Cooked rind.

References

- Egelandsdal B., Kidman S. and Hermansson A. -M: 1991 In: Food Structure 10, 303-316
- Katsaras K. and Peetz P.: 1988 In: Fleischwirtschaft 68, 1187-1192
- Kotter L. and Prändl O.: 1958 In: Fleischwirtschaft 10, 26-28
- Laemmli. U.K.: 1970 In: Nature 227, 680-685
- Lehninger A.:. 1975 In: Biochemistry ed. A. Lehninger, Worth Publ. NY, 10016 USA
- Towbin H.J., Staehlin Th. and Gordon J.: 1979 In: Proc. Natl. Acad. Sci. USA, 76, 4350-4354
- Zijderveld Monique H.G. and Koolmees P.A.:1990 In: Meat Sci 27, 5