HPLC-MS/MS-DETECTION OF TRANSGLUTAMINASE IN FORMED BEEF AND PORK

Wolfgang Jira and Fredi Schwägele

Department of Safety and Quality of Meat, Max Rubner-Institut (MRI), 95326 Kulmbach, Germany

Abstract – A sensitive HPLC-MS/MS method for the detection of transglutaminase (TG) from *Streptomyces mobaraensis* in formed beef and pork was developed, using tryptic marker peptides. After optimization of the extraction conditions and tryptic digestion formed meat and blank values were analyzed in a raw and heated state. By investigation of samples pre-treated with marinades, seasoning salt and breadcrumbs only very little effects of the type of pre-treatment on the detectability of TG were found. Using four marker peptides no false-positive or false-negative results were obtained. The limit of detection (LOD) was about a factor of 10 below the recommended amount of TG.

Key Words – formed meat, transglutaminase, tryptic marker peptides

I. INTRODUCTION

Transglutaminase (TG) from *Streptomyces mobaraensis* can be used for restructuring large segments of intact looking meat by binding together small pieces of meat. In this context the safety aspects consumer deception, possible problems for people suffering from coeliac disease [1,2] and the translocation of microbial contaminations into the interior [3] are currently under discussion.

The main objective was to develop an analytical method for the mass spectrometric detection of TG in formed beef and pork, using characteristic tryptic marker peptides. The formed meat samples were analyzed as raw meat as well as after thermal processing. The influence of pre-treatment with marinades, seasoning salt, and breadcrumbs on the detectability of TG was also investigated.

II. MATERIALS AND METHODS

Meat Binding experiments with beef and pork

Fresh pork (Longissimus dorsi) and beef (roast beef) were cut into meat pieces (2 – 2.5 cm). To 1 kg of meat cubes 5 g of salt (NaCl) and 2 g of phosphate were added and the meat pieces were tumbled in a vacuum tumbler for 30 min at 2-4 °C. Suspensions of Activa EB (Ajinomoto) were evenly distributed on the surface of the tumbled meat pieces. Meat pieces were put in bags under vacuum and were stored for 17-25 hours at 2-4 °C. The restructured meat was cut into slices (1.5 cm) and either directly used for TG analysis or for frying experiments. Before frying some samples were pre-treated with oil marinade (OM), emulsion marinade (EM), seasoning salt (SS), as well as breadcrumbs (BC). For each type of meat binding experiment meat slices of the same piece of meat with comparable thickness and weight without addition of TG were used as blanks (Tab. 1).

Table 1 Meat Binding experiments and blanks

Activa EB [%]	N	Processing	Species	Treatment
0.8/---	4/4	raw/heated	Beef/pork	---
0.8/---	2/2	heated	Beef/pork	OM
0.8/---	2/2	heated	Beef/pork	EM
0.8/---	2/2	heated	Beef/pork	SS
0.8/---	2/2	heated	Beef/pork	BC
1.2/---	2/0	raw/heated	Pork	---
0.8/---	2/0	raw/heated	Pork	---
0.5/---	2/0	raw/heated	Pork	---
0.2/---	2/0	raw/heated	Pork	---
0.1/---	2/0	raw/heated	Pork	---

Grilling and frying experiments

Grilling experiments were performed with a Silex S-162 contact grill. The upper and lower grill plate were preheated to 170 °C. A slice of meat (blank) or formed meat, wrapped in aluminium foil, was fried for 3 min and cooled to room temperature (RT). The frying experiments with breaded meat (formed meat and blank) were performed in a stainless steel pan with 30 g rapeseed oil, preheated to 180 °C. A slice was fried for 3 min, turned and fried for about 6 min, until a core temperature of 72 °C was reached. The fried meat slices were allowed to drain off oil and cooled to RT.

LC-MS/MS-Detection of transglutaminase

Homogenized meat was extracted with acetone and dried at RT. 50 mg of the defatted and dehydrated sample were shaken in 0.5 mL TRIS-HCl (1M, pH 8.2) for 2 h at 100 °C, cooled to RT and centrifuged. To a 100 µL sample of the protein extract 2 ng Trypsin were added and incubated at 37 °C for 3 h. After addition of 2 µL conc. formic acid (FA) the sample was cleaned up on a Strata-X SPE column (30 mg; elution with 0.5 mL 90% ACN in water in a 1 mL tapered vial, prefilled with 5 µL DMSO [4]), concentrated to 5 µL and dissolved in 50 µL of solvent A (97% water, 3% ACN, 0.1% FA).

Separation of peptides was performed with a Dionex UltiMate 3000 RS HPLC (Nucleosil 100-3 C18 HD; 125 x 2.0 mm) using a gradient elution with water/ACN (0.1% FA) at 40°C (injection volume: 10 µL) and a flow rate of 250 µL/min. Peptide detection was carried out on an AB Sciex QTrap 5500 using the following parameters: Source temperature: 430 °C, ion spray voltage: 5.5 kV, curtain gas flow: 35. Details of the scheduled Multiple Reaction Monitoring (MRM) method are shown in Table 2.

Table 2 Parameters of the MRM method

Marker	m/z	Product ions	CE
1	589.8 (+2)	978.5/778.4/679.4	28/30/28
2	453.2 (+2)	677.3/606.3/420.2	24/22/20
3	554.8 (+2)	490.8/817.5/605.3	20/22/20
4	585.8 (+2)	986.4/839.4/768.3	25/28/28
5	470.7 (+2)	756.4/609.3/446.3	24/25/25
6	547.8 (+2)	447.7/797.4/500.3	24/31/39

III. RESULTS AND DISCUSSION

The meat binding experiments using 0.8% Activa EB applying a TG exposure time of at least 17 hours led to satisfying results. In Fig. 1 a beef steak (blank) and a restructured beef (Activa EB) are shown.

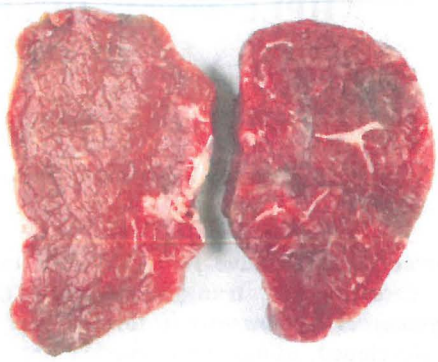

Fig. 1. Beef steak (Blank; left) and restructured beef (Activa EB; right)

On the basis of HPLC-MS/MS measurements of the tryptic digest of Activa WM (no additional protein ingredients) using the maXis UHR-QToF system and subsequent submission of the obtained peak lists to the online version of the MASCOT database search tool (www.matrixscience.com) the six tryptic marker peptides AETVVNNYIR (1), EVASVMNR (2), GAYVITFIPK (3), LAFASFDEDR (4), SPFYSALR (5), and VTPPAEPLDR (6) were obtained.

The uniqueness of these peptides was checked by database searching using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and UniProt (http://www.uniprot.org/blast/). Besides their occurrence in TG of *Streptomyces mobaraensis* these peptides are also found in TG of various Streptomyces subspecies. Furthermore all six TG marker peptides occurred in TG of *Escherichia coli*. Marker peptide 2 was also found in the formate/nitrite transporter (FNT) of *Paenibacillus panacisoli*. The mentioned homologies of the TG marker peptides were classified as unproblematic due to their irrelevant occurrence in meat and spices.

For the optimization of the extraction conditions, samples of TG treated pork (0.8% Activa EB; raw and heated) were extracted for 2 h at different extraction temperatures (60 °C, 70 °C, 80 °C, 90 °C, and 100 °C). The best results were observed at an extraction temperature of 100 °C for raw as well as grilled material. For the tryptic digestion different times (1 h, 2 h, 3 h, 6 h, and 18 h) were tested. It was shown that a digestion time of 3 h led to better results compared to 18 h for two marker peptides (1 and 3). For two marker peptides (4 and 6) the peak areas were lower applying a digestion time of 3 h compared to 18 h.

With respect to the disadvantages of a digestion time of 18 h and the aim of the study to develop a rapid method a digestion time of 3 h has been chosen.

A chromatogram of the six TG marker peptides in fried formed pork pre-treated with OM is shown in Fig. 2.

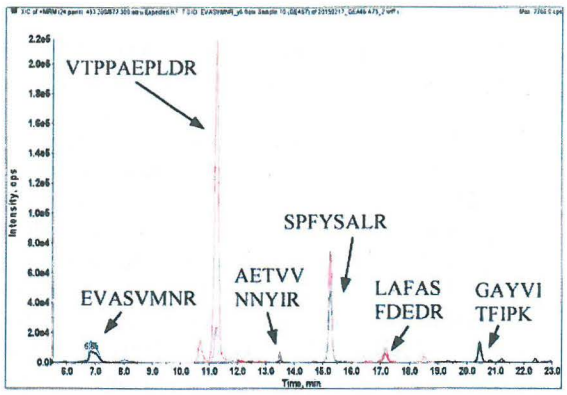

Fig. 2. Chromatogram of the TG marker peptides in formed fried pork (0.8% Activa EB)

The detection of a TG marker peptide was considered to be positive, if all three mass transitions (see Table 2) showed a signal-to-noise ratio (S/N) of at least 3 (limit of detection; LOD). Under these conditions in the blank values with the exception of SPFYSALR the TG marker peptides were not detected. Small amounts of SPFYSALR in the range of the LOD (average S/N ratios in the range of 3 to 19) were detected in most of the blank values of beef and pork. Therefore SPFYSALR is suitable as TG marker peptide to only a limited extent, even significant differences in the peak areas and S/N ratios between TG treated samples and blank values were observed. It was shown that the measurement of three mass transitions for each marker peptide enables a more reliable detection of a peptide compared to the measurement of two mass transitions: For the marker peptide EVASVMNR in some blank values of beef and for GAYVITFIPK in blank values of pork low peak areas of two mass transitions were observed. With the help of the third mass transition it was clearly possible to exclude the presence of these marker peptides in the mentioned blank values.

In all meat binding experiments with beef and pork (0.8% Activa EB) all six marker peptides were present. The limiting factor for the detection of a marker peptide is the S/N ratio of the mass transition with the lowest S/N ratio. Considering the TG marker peptides, for which no false positive results were observed (marker 1, 2, 3, 4, and 6) the S/N ratios of the mass transition with the lowest S/N ratio ranged between 15 ± 3 (AETVVNNYIR) and 98 ± 23 (VTPPAEPLDR) (N=6) and were consequently a factor of 5 to 33 above the LOD.

When comparing the detectability of TG in restructured meat pre-treated under various conditions (raw, heated, OM, EM, SS, BC), no significant differences between the different treatments were observed. It was shown that grilling and frying and also the pre-treatment of samples before heating did not adversely affect the detectability of TG.

The suggested amount of Activa EB for restructuring of meat is 1% [5]. For an estimation of the LOD of TG in formed meat, meat binding experiments with different amounts of Activa EB were performed (see Table 1). It was shown, that the use of 0.1% and 0.2% Activa EB did not lead to satisfactory results: Within these experiments the meat pieces fell apart during slicing.

Samples of each batch (0.1, 0.2, 0.5, 0.8, and 1.2% Activa EB) were analysed in three-fold determination in raw and heated state. For samples of raw formed meat for the four marker peptides satisfying correlations (R^2 between 0.95 and 0.99) between the lowest S/N ratio of the three mass transitions and the amount of Activa EB were observed (Fig. 3).

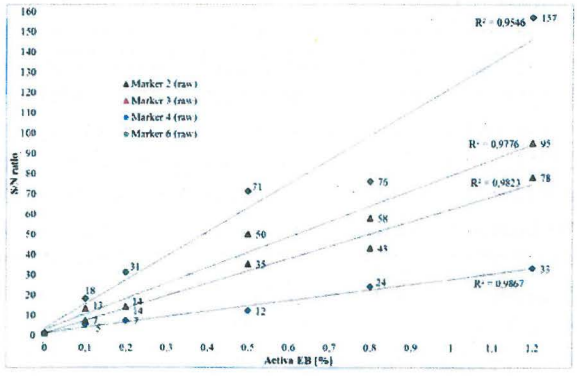

Fig. 3. Correlation between the lowest S/N ratio of the three mass transitions of marker 2, 3, 4, and 6 (mean values) and the amount of Activa EB [%] in raw formed meat

For an added amount of 0.1% Activa EB all four marker peptides showed S/N ratios greater than 3 (between 5 and 18). Consequently all four marker peptides showed LODs below 0.1% Activa EB. However, the determined S/N ratios showed relatively high percentage standard deviations, which ranged between 0% and 62% at a concentration of 0.1% Activa EB. For higher concentrations of Activa EB the percentage standard deviations ranged between 4% and 43%. For marker peptide 3 the detectability in raw and heated formed meat is very similar. For the other marker peptides the detectability in heated formed meat is slightly worse. Nevertheless, for marker 2, 3, and 6 LODs of 0.1% Activa EB were achieved. The LOD of marker 4 was between 0.1 and 0.2% Activa EB. The percentage standard deviations of the S/N ratios were higher than the corresponding values observed for raw formed meat and ranged between 0% and 67% at a concentration of 0.1% Activa EB.

IV. CONCLUSION

By the selection of four characteristic tryptic marker peptides (EVASVMNR, GAYVITFIPK, LAFASFDEDR, and VTPPAEPLDR) TG from *Streptomyces mobaraensis* could be reliably detected in formed meat, using amounts of Activa EB 20% below the recommended dosage of 1%. Heat processing like grilling or frying as well as different pre-treatments of formed meat showed no remarkable influence on the detectability of TG in formed meat. The LODs for the four mentioned marker peptides in raw restructured meat were 0.1% Activa EB. For heated formed meat with the exception of the marker peptide LAFASFDEDR (LOD between 0.1% and 0.2%) also LODs of 0.1% Activa EB were achieved. Consequently the LODs were a factor of 10 below the recommended amount of TG (1%) and thus in a scale, in which no binding efficiency was achieved any more.
In further studies, it shall be clarified, if the present screening method for the mass spectrometric detection of TG in formed meat is also suitable for the detection of TG in formed meat products with long maturing times such as dry-cured formed ham. In this context it should be investigated, whether or to what extent the detectability of TG decreases during the ripening process due to the activity of proteolytic enzymes.

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