HPLC-MS/MS-DETECTION OF FIBRINOGEN/THROMBIN IN FORMED BEEF AND PORK

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Abstract – A sensitive HPLC-MS/MS method for the detection of bovine and porcine fibrinogen/thrombin in restructured meat was developed using tryptic marker peptides of bovine and porcine fibrinogen (six markers each). Meat binding experiments with beef and pork were performed using bovine and porcine blood-based plasmapowder FG (PPFG; Sonac B.V.). The method developed allows the detection of the use of these cold-set binders in raw and heated samples. The peak areas of the fibrinogen marker peptides were increased by a factor of about 100 compared to blank values originating from the occurrence of residual blood in meat using a concentration of 0.6% bovine and porcine PPFG.

Key Words – restructured meat, bovine and porcine fibrinogen, tryptic marker peptides

I. INTRODUCTION

The protein-based binding systems bovine and porcine plasmapowder FG (PPFG; Sonac B.V., Netherlands) for the restructuring of meat are commercially available in powder form and used as cold-set binders [1]. The blood-based PPFG utilizes the blood clotting mechanism [2] and contains a strongly increased fibrinogen concentration, which is achieved by a patented process [3].
The main objective was to develop an analytical method for the simultaneous mass spectrometric detection of bovine and porcine PPFG in formed beef and pork, using characteristic tryptic marker peptides. The formed meat samples and corresponding control samples were analyzed as raw meat as well as after thermal processing.

II. MATERIALS AND METHODS

Meat Binding experiments with beef and pork
Fresh beef (Biceps femoris) and pork (Longissimus dorsi) were cut against the meat grain into slices (thickness 8 mm; weight about 50 – 80 g). The upper sides of two slices were sprinkled with PPFG (0.2, 0.6 and 1.0%) or were not powdered (control samples). PPFG from porcine plasma were used for pork and PPFG from bovine plasma were used for beef (Tab. 1).

Table 1 Meat Binding experiments and blanks

Experiment	N	Binding Agent [%]	Beef	Pork
Blank	24	---	+	+
Bovine PPFG	10	0.2	+	---
Bovine PPFG	10	0.6	+	---
Bovine PPFG	10	1.0	+	---
Porcine PPFG	10	0.2	---	+
Porcine PPFG	10	0.6	---	+
Porcine PPFG	10	1.0	---	+

The two meat slices were superposed and wrapped in cling film. The cling film was dipped in at several points before storage for about 20 h at 2 to 4 °C. The formed meat slices were used either for the HPLC-MS/MS analysis or for the grilling experiments.

Performance of the grilling 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 4 min and cooled to room temperature (RT).

HPLC-MS/MS-Detection of bovine and porcine fibrinogen
Homogenized meat was extracted with acetone and dried at RT [4]. 50 mg of the defatted and dehydrated sample were shaken in 0.5 mL TRIS-HCl (1M, pH 8.2) for 1 h at 100 °C, cooled to RT and centrifuged. To a 100 µL sample of the protein extract 2 µg 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 [5]), 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
BOV1	531.3 (+2)	877.4/764.4/635.3	24/23/28
BOV2	487.3 (+2)	731.4/618.3/505.2	20/22/20
BOV3	743.4 (+2)	973.5/584.3/513.3	27/33/32
BOV4	560.8 (+2)	836.4/721.3/606.3	27/30/33
BOV5	595.3 (+2)	861.4/748.4/495.3	29/28/24
BOV6	613.3 (+2)	950.5/851.4/764.4	32/30/30
PIG1	616.0 (+3)	767.4/710.8/667.3	22/24/21
PIG2	704.9 (+2)	598.8/525.3/360.2	28/32/30
PIG3	554.3 (+2)	850.4/735.4/606.3	30/30/31
PIG4	575.7 (+3)	760.5/647.4/324.2	27/23/21
PIG5	707.4 (+2)	650.8/607.3/502.3	28/24/32
PIG6	790.5 (+2)	883.5/699.4/359.2	38/34/30

III. RESULTS AND DISCUSSION

Sufficient results of the meat binding experiments were obtained for raw meat treated with 0.6 and 1.0% PPFG. The binding efficiency was lower for raw meat treated with 0.2% PPFG. The grilled meat samples treated with 0.6 and 1.0% PPFG showed a strong binding and the grilled samples treated with 0.2% PPFG showed a sufficient binding.
On the basis of HPLC-MS/MS measurements of the tryptic digests of bovine and porcine PPFG using the maXis UHR-QToF system and subsequent submission of the obtained peak lists to the MASCOT database search tool (www.matrixscience.com), six marker peptides for bovine (ALLEMQQTK (BOV1), ELLIDNEK (BOV2), GDSVSQGTGLAPGSPR (BOV3), GNLDDFFHR (BOV4), ISQLTNMGPTK (BOV5), QFVSSSTTVNR (BOV6)) and porcine fibrinogen (AIQISYNPEDLSKPDR (PIG1), DPFPDFFSPVLK (PIG2), GSLDEFFHR (PIG3), IHLITTQSAIPYVLR (PIG4), ISPLPDITPADFK (PIG5), QLDQVIAINLLPSR (PIG6)) were obtained.
The uniqueness of these marker peptides was checked by database searching using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and UniProt (http://www.uniprot.org/blast/). All marker peptides for bovine fibrinogen were specific for fibrinogen, except for BOV2, which can also occur in hypothetical proteins of microorganisms, however, various homologies to fibrinogen from other species were observed. In comparison to the marker peptides for bovine fibrinogen, significantly fewer homologies were observed

for the marker peptides for porcine fibrinogen selected. Only PIG3 showed homologies to fibrinogen of different camel species.
Chromatograms of the marker peptides of bovine and porcine fibrinogen in formed raw meat (0.6% PPFG) are shown in Fig. 1.

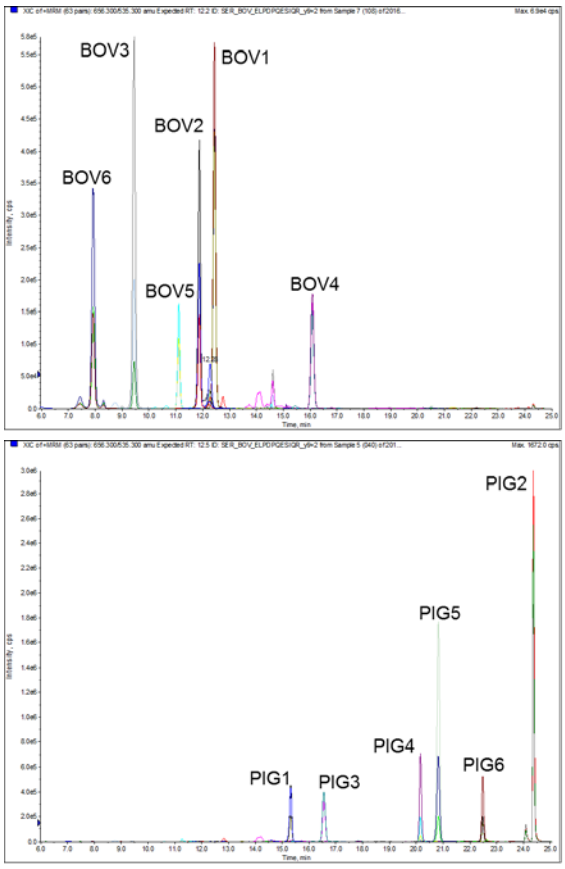

Fig. 1. Chromatograms of raw beef and pork, restructured with 0.6% bovine and porcine PPFG
A clear differentiation between the use of bovine and porcine PPFG is already possible based on the MRM chromatograms by the selection of six marker peptides each for bovine and porcine fibrinogen: Whereas the six marker peptides for bovine fibrinogen show HPLC retention times between 8 and 16 min, the marker peptides for porcine fibrinogen have retention times between 15 and 24 min.
The fact that meat always contains a certain amount of residual blood and, consequently, fibrinogen has to be considered regarding a reliable detection of the use of fibrinogen/thrombin for restructuring of meat of the same animal species. Therefore, at least, comparative investigations of control samples are required to detect the use of fibrinogen/thrombin. Concerning this aspect, theoretical calculations should be performed in the following regarding which amounts of fibrinogen are to be expected in untreated meat (blank values) and in meat restructured with PPFG. In agreement with the literature, meat contains about 2 to 9 mL residual blood per kg muscle, corresponding to a mean value of 5.5 mL/kg [6]. Assuming a percentage of 65 to 70% (mean: 67.5%) plasma to the total volume of blood [7], the mean content of plasma in meat is about 3.5 mL/kg. With a proportion of 0.4% fibrinogen in plasma [8], a content of about 14 mg fibrinogen per kg arises for untreated meat (blank value). The commercially available PPFG contains no information on the amount of fibrinogen and merely a protein content of 70% is specified. Since PPFG is a dried form of the product “Fibrimex”, showing a protein content of 9.5% and a fibrinogen content of 5% [9], a percentage of fibrinogen of 37% in PPFG can be calculated based on the percentage of fibrinogen of 53% of the total protein content. By adding the recommended amount of PPFG in the range of 0.4 to 0.7%, this corresponds to added amounts of fibrinogen in the range of about 1480 to 2590 mg/kg. This is equal to increases of the fibrinogen

concentrations by a factor of about 106 to 185. These theoretically calculated increases of the fibrinogen concentrations by about two orders of magnitude suggest that a mass spectrometric detection of the use of this binding system by means of comparative investigations of untreated meat (blank values) and restructured meat using PPFG is quite possible.

Significantly increased peak areas compared to the control samples have already been observed for all six marker peptides for bovine fibrinogen after an addition of 0.2% bovine PPFG. The mean values of the peak areas in beef samples formed with 0.2% bovine PPFG were about a factor of 21 (BOV2 and BOV4) to 50 (BOV6) higher than in the control samples (Tab. 2). Differences in the peak areas between control samples and samples with 0.2% porcine PPFG in the same order of magnitude (between a factor of 32 (PIG2 and PIG3) and 46 (PIG5)) were observed for restructured pork. By adding the recommended amount of PPFG (0.6%), the factors, by which the peak areas of the fibrinogen marker peptides were raised in comparison to the blank values were in the range of 63 to 151 (mean: 90) and from 100 to 148 (mean: 123) for beef and pork, respectively. These factors correspond roughly to increases of two orders of magnitude compared to control samples, confirming the theoretical calculations performed.

Table 2 Factors (N = 10), by which the peak areas of the fibrinogen marker peptides are raised for samples with different amounts of added PPFG

	Peak area ratio compared to control samples		
	0.2% PPFG	0.6% PPFG	1.0% PPFG
Beef (N=10)			
BOV1	26	84	143
BOV2	21	65	113
BOV3	34	109	188
BOV4	21	63	109
BOV5	22	68	120
BOV6	50	151	257
Mean value	*29*	*90*	*155*
Pork (N=10)			
PIG1	38	127	195
PIG2	32	100	146
PIG3	32	101	158
PIG4	45	148	249
PIG5	46	129	210
PIG6	41	130	214
Mean value	*39*	*123*	*195*

Fibrinogen may be degraded during the thermal processing of restructured meat. Therefore, the peak areas of the fibrinogen marker peptides of heated samples were compared to raw samples separately for the three concentrations of PPFG applied (0.2, 0.6 and 1.0%, respectively). The peak areas of bovine and porcine fibrinogen marker peptides in heated material ranged from 58 to 122% of those in raw material. Therefore, all fibrinogen marker peptides could also be sufficiently detected in heat material, even for samples with 0.2% PPFG.

IV. CONCLUSION

The analytical method developed allows an easy differentiation between the application of bovine or porcine fibrinogen and can be fast performed due to the short times for protein extraction (1 h) and tryptic digestion (3 h). The purely qualitative detection of fibrinogen is insufficient for the evidence of the use of fibrinogen/thrombin due to the occurrence of residual blood in meat. Because the peak areas of the fibrinogen marker peptides are highly increased in restructured meat compared to blank values (0.6% PPFG: about a factor of 100; 0.2% PPFG: about a factor of 30 to 40), evidence of the use of this binding system based on comparative investigations is easily possible even for concentrations of PPFG, in which the binding efficiency in raw restructured meat was just sufficient. In this context, it should be noted that comparative investigations

cannot be avoided by the selection of marker peptides which are present in only very low concentrations such as bovine and porcine fibrinopeptide A and B [10,11], because a differentiation between positive and negative results is more difficult for concentrations in the range of traces compared to higher concentrations.

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