Analytical Tools for Rapid, Sensitive, Quantitative Identification of Potential Meat Quality Markers

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Abstract. Myofibrillar extracts from bovine *Musculus longissimus dorsi* (MLD) were subjected to SDS-PAGE, electroblotted and fragments of the 30 kDa band determined by internal and N-terminal Edman sequencing, giving unequivocal proof, troponin-T (TNT) to be the origin of this band. Based on the N-terminal primary sequence of the 30 kDa band, a peptide with high antigenic sites was synthesized, conjugated to keyhole limpet hemocyanin (KLH), antibodies were generated and an enzyme-linked immunosorbent assay (ELISA) was developed for the determination of TNT concentrations in meat samples. For routine determinations of meat quality markers it seems more convenient to analyse soluble meat extracts, produced by trichloroacetic acid (TCA) or HCl treatment. In the supernatants of TCA-treated MLDs, prominent peptide fragments from glyceraldehyde-3-phosphate dehydrogenase and TNT (16–31) could be separated by HPLC and identified by Edman degradation. Both fragments were found to increase with ageing and might become useful indicators of meat quality. After HPLC separation and structure elucidation of MLD HCl extracts, fructose–biphosphate aldolase, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase and myoglobin could be identified, further potential candidates to correlate their quantitative appearance with meat quality. These peptides and proteins, found in soluble meat extracs, can be analyzed in an automatic, rapid, convenient way either by immunoassay methods, capillary electrophoreses or HPLC, for sure preferable compared to the tedious, inconvenient, time-consuming method of SDS-PAGE, also not suitable for automation.

Conversion of muscle tissue to meat, including tenderization, is a complex process. As the consumer considers meat tenderness to be the most important criterion for meat quality, this conversion should be elucidated on a biomolecular basis. With such a biochemical background, specific sensitive analytical tools could be developed which would correlate physicochemical parameters with the degree of tenderness.

Various events take place at the level of the myofibrils which have been correlated with meat tenderness. These include *Z*-disk weakening [1], degradation of desmin or titin, leading to fragmentation or weakening of myofibrils, the disappearance of troponin-T and simultaneous appearance of 28–32 kDa proteins or detection of a 110 kDa protein fragment, as monitored thoughout the conditioning period by electron microscopic studies and SDS polyacrylamide gel electrophoresis, respectively [2–7]. In particular, the disappearance of troponin-T and the concomitant appearance of 28–32 kDa proteins seem to be good indicators of *post-mortem* proteolysis. However, the origin of the 30 kDa fragments was speculative and not determined on a structural basis [8–10]. Therefore, we recently applied the technique of internal microsequencing to provide for the first time unequivocal proof for the origin of the 30 kDa protein [11]. The 30 kDa protein transferred to ProBlott and Immobilon membranes was digested with trypsin, the peptide mixture separated with HPLC, selected peptides isolated and sequenced. Eight peptides, varying from 5 to 13 amino acid residues, displayed between 50 and 100% homology to rabbit troponin-T, according to our protein homology search. As SDS-PAGE operations are tedious, inconvenient and time-consuming, we suggest in this communication

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quantification of meat quality markers applying immunoas-says, high performance liquid chromatography (HPLC) or capillary electrophoresis (CE), allowing high sample throughputs and suitable for automation.

Experimental

Preparation of Samples

For Electroblotting

Sections of *Musculus Longissimus dorsi* (MLD) were excised from Hereford cross heifers at 24 h *post-mortem* and aged at 3 ± 1 °C for 2, 7 and 14 days. The muscle was trimmed of fat and connective tissue, and finely chopped. Meat samples are placed in capped tubes, frozen in liquid nitrogen and stored at –70 °C. Myofibrillar proteins were extracted from these samples using the method developed by Wang [12].

The freeze-dried myofibrillar proteins were concentrated further by dissolving them at a concentration of 4.33 mg/mL of 0.5M Tris, pH 6.8, vortexing and mixing overnight. The samples were centrifuged at 8.720 × g for 5 min and 1.5 ml of the supernatant was freeze-dried. The residue was resuspended in approximately 150 µl of sample buffer for SDS-PAGE.

For Trichloroacetic Acid Extraction

Hereford cross heifers were slaughtered conventionally, the MLDs excised from the carcass at 1 h *post-mortem* and stored at 4 °C for 14 days. At 1 h, 1 d, 3 d and 14 d *post-mortem* MLD samples of approximately 60 g weight were excised and extractions carried out at 4 °C as follows: All exposed surfaces were removed from the MLD, 50 ml of distilled H_2O added to 25 g of meat and homogenized in an Omni mixer (Omni International, Waterbury, Connecticut, USA) as follows: 30 sec at speed 4, then 15 sec rest period and finally 30 sec at speed 4. The blades of the Omni mixer were scraped down to include all connective tissue in the homogenate. Then 8.33 mL of 50% TCA were added to this homogenate to bring the final trichloroacetic acid (TCA) concentration to 5%. The homogenate was mixed well, transferred to a centrifuge tube and subsequently spun at 2 958 × g for 30 min in a Mistral 3 000 centrifuge (Crawley, Sussex, England). The supernatant was filtered through cheesecloth X 8, divided into aliquots in 1.5 mL Eppendorf tubes and 5 mL screw top tubes and stored at –20 °C.

For HCl Extraction

In a similar way, HCl extracts of MLD samples were prepared except that 25 g of meat samples were homogenized with 50 ml of 0.01N HCl, and then 8.33 mL of water were added to the homogenate.

For TBS Extraction

Whole muscle samples from bovine MLD were frozen in liquid nitrogen and homogenized in a steel waring blendor (New Hartford, Connecticut, USA), followed by homogenisation in Tris-buffered saline (TBS) buffer, pH 8.0 (Sigma, Deisenhofen, Germany; 0.2 g/20 mL), and centrifugation at 8 720 × g for 10 min. Aliquots of the supernatant were prepared and frozen at –20 °C.

SDS-PAGE Electrophoresis and Electroblotting

SDS-PAGE of the samples to be prepared for electroblotting was carried out according the method of [13], using a 3.0% stacking and a 15% separating gel. High range SDS-PAGE protein standards (BIO-RAD, Munich, Germany) were used as molecular weight markers. Separation was run over 4 hours at a constant voltage of 165 V (Consort E 831 power box, Delmar, CA, USA). The resulting protein bands were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (ProBlott™ from Applied Biosystems, Weiterstadt, Germany) using the method published in [14]. Transfer was achieved using 0.1M CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer, pH 11, with 10% methanol and a constant power of 20 V over night (approximately 16 hours). PVDF blots were stained with Coomassie Blue G dye (Sigma, Deisenhofen, Germany) and destained with 100% methanol followed by 50% methanol, washed in destilled water and dried in air.

High Performance Liquid Chromatography

Samples, collected after TCA and HCl extraction, respectively, were separated by reversed-phase high performance liquid chromatography (RP HPLC). The HPLC system (Eppendorf, Hamburg, Germany) consisted of two Model BT 810 pumps with a controller for gradient programming and a Rheodyne injection valve BT 8121 with a 500 µl loop. The separations were carried out on LiChrospher 100 RP-18 (5 µm; 250 mm × 4 mm i.d.; Merck Eurolab GmbH, Darmstadt, Germany) or Nucleosil 100 RP-18 (7µm; 250 mm × 10 mm i.d.; Macherey-Nagel, Düren, Germany) columns with a H_2O/TFA(trifluoroacetic acid)/CH_3CN gradient system. More detailed chromatographic conditions are given in the legends of the figures. After collection, the peptide fractions were lyophilized and further analysed by mass specrometry and automatic Edman sequencing.

Sequencing Analysis

Automated Edman degradation was performed using an Applied Biosystems pulsed liquid sequencer model 473A (Weiterstadt, Germany) with on-line analysis of the phenylthiohydantoin (PTH) derivatives. The electroblotted protein bands of interest were excised from the PVDF-blotted membrane using a razor blade. The membrane pieces were activated by wetting in 100% methanol, followed by 20% methanol. The membrane pieces were washed with distilled water, cut into smaller pieces and inserted into the slot of the sequencer cartridge. The sequence of the unknown protein was read by comparing the HPLC chromatograms of standard PTH-derivatized amino acids with those produced by sequential Edman degradation of the blotted protein fragment. Depending on the results obtained, 20–30 Edman degradation cycles were used. For sequence determination of peptides and proteins isolated *via* HPLC from meat samples after TCA or HCl extraction, approximately 50–200 pmol sample were applied on the cartridge filter, previously treated with polybrene, as described in [15] and [16].

Laser Desorption Mass Spectrometry

For mass determination, a laser desorption mass spectrometer (LDMS; Kratos Kompact MALDI I) from Shimadzu (Duisburg, Germany) was applied, as described in detail in [17] and [18]. The peptide sample (0.2 µL) was mixed with 0.3 µL of matrix solution and applied on the sample slide. The droplet was allowed to dry and then the target was loaded for analysis into the instrument. α-Cyano-4-hydroxycinnamic acid from Sigma (Deisenhofen, Germany), dissolved in 70% acetonitrile and 0.1% TFA in water, was used as matrix. The concentration of matrix ranges between the point of saturation and one third of that concentration.

Capillary Electrophoresis

Capillary electrophoretic separations were performed on a Bio-Rad (Munich, Germany) BioFocus 3000 CE system, equipped with an automatic constant volume sample injection system, a temperature control system for the capillary, sample and fraction collection compartment, a high sensitivity fast-scanning UV-VIS detector with wavelength programming and a dedicated computer system with a Microsoft Windows interface. During all runs, the capillary and the sample compartment were cooled to 15 °C. For more experimental details see [19 – 22].

Search for Protein Homologies

Comparison of the sequences obtained with protein and DNA-derived protein sequence databases was carried out using an on-line connection to the European Molecular Biology Laboratory (EMBL, Heidelberg) and MEDLINE links with the BLAST database *via* the Internet.

Antigenic Site Prediction

A computer program, developed by [23], integrating hydrophilicity (H), surface probability (S), backbone flexibility (F) and secondary structure (CF: Chou-Fasman; GR: Garnier-Robson) parameters, is used to predict potential antigenic sites of peptides and proteins and applied as described in detail by Voelter *et al*. [24 – 26].

Peptide Syntheses

The peptides were produced by the solid phase method using an Eppendorf (Hamburg, Germany) ECOSYN P automatic batch synthesizer [27]. Briefly, the synthesis started with 1.0 mmol $^{\alpha}$*N*-Fmoc-protected aminoacid residue-*p*-benzyloxybenzyl alcohol resins, respectively, (Bachem, Switzerland), provided with a loading of 0.6 mmol/g. The α-amino functions of all amino acids used were protected by the fluorenylmethoxycarbonyl (Fmoc) group. The couplings were performed using a fourfold excess in protected amino acids and coupling reagent (0.13 g TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) plus 1.2 ml diisopropylethylamine (DIEA; 12.5% solution in DMF). Before coupling the protected amino acids, the Fmoc groups were removed from the amino acid and peptide resins, respectively using 25% piperidine in DMF. Double couplings were performed for Fmoc-Gln(Ttr)-OH and Fmoc-Ile-OH. After cleavage of the N-terminal Fmoc group, the peptides were removed within 3 h at room temperature from the resins under simultaneous cleavage of the amino acid side chain-protecting groups using a mixture of TFA/thioanisol/anisol/water/triisopropylsilane. The mixtures were filtered, washed with TFA and the combined filtrates precipitated with anhydrous ether. Desalting was performed by gel chromatography and 5% acetic acid elution on a TSK-HW 40 S column (100 mm × 1.6 mm i.d.; TosoHaas, Montgomeryville, PA, USA) and further purification was performed *via* HPLC and a Nucleo-sil 100 C18 column. Further chromatographic conditions are given in the legends to the figures. The purities of the compounds were controlled by analytical HPLC and the structures confirmed by LDMS.

Production of Polyclonal Antibodies against Synthetic Peptide Fragments of Troponin-T

The synthetic peptides, rabbit TNT (34–62) [*i.e.*, the 30 kDa sequenced fragment] and bovine TNT (16–31) [*i.e.*, the bovine TNT soluble fragment] were coupled to an equal amount of soluble keyhole limpet hemocyanin (KLH, Sigma, Deisenhofen, Germany), dissolved in 0.1M phosphate buffered saline (PBS), pH 7.2, (Sigma, Deissenhofen, Germany), using 100–200 µL of 0.3% glutaraldehyde (Sigma, Deisenhofen, Germany) as linking agent. The reagents were allowed to react for 2–3 hrs at room temperature under continuous stirring and unreacted glutaraldehyde was further blocked with 1M glycine. The mixture was dialysed against 1 L of PBS or borate buffer, pH 8.5, over night at 4 °C according to the methodology previously described [28]. Two male white New Zealand rabbits received primary immunizations by subcutaneous injections of 0.5 mg of TNT (34–62) or TNT (16–31), emulsified with complete Freund´s adjuvant per rabbit per immunization, as described in [29]. Antisera were collected 7–10 days after the third and subsequent immunizations, boosters carried out at 4-week intervals.

ELISA Protocols

For the development of the relevant immunoassays, indirect ELISAs were set using two protocols [30]:
Protocol I. Flat bottom 96-well EIA plates were coated with 100–0.1 ng/well of the respective peptide used for immunizations, *i.e.*, TNT (34–62) or TNT (16–31), and with the unknown samples serially diluted (1:1 to 1:10 000) in coating (carbonate pH 9.6) buffer. Plates were incubated over night at 4 °C and subsequently washed and blocked (1 h at 37 °C) with Tris-buffered saline (TBS) containing 3% milk (M-TBS). Anti-TNT (34–62) or anti-TNT (16–31) (diluted 1:10 000 in M-TBS; 100 µL/well) were added and incubated for 2 hrs at 37 °C. After washing with M-TBS, the secondary antibody (Ab; donkey or goat anti-rabbit total IgG-horseradish peroxidase [HRP] conjugate; diluted 1:1,000 in M-TBS; 100 µL/well) was added, followed by incubation (1 h at 37 °C), washing with M-TBS and finally with TBS. As substrate, *o*-phenylenediamine dihydrochloride (OPD; diluted in phosphate-citrate buffer, pH 5,0, (Sigma, Deisenhofen, Germany); 100 µL/well) was used. After 20 min incubation at ambient temperature, the reaction was terminated with 3N HCl and the absorption read at 490 nm.

Protocol II. Similarly, coated EIA plates were incubated at

37 °C for 1 h and over night at 4 °C. After washing with PBS, blocking was performed with PBS-Tween 20 (0.1%)- bovine serum albumin or bovine serum albumin (BSA; 1%) or bovine serum (BS; 5%) (30 min at 37 °C; 150 µL/well), followed by PBS-Tween 20 washing. The primary Ab (diluted 1:10 000 in PBS-Tween 20-BSA or BS; 100 µL/well) was added and incubation continued for 90 min at 37 °C. Wells were washed again with PBS-Tween 20, the same secondary antibody-HRP conjugate as in protocol I was added (diluted 1:1000 in PBS-Tween 20-BSA or BS; 100 µL/well), followed by incubation (1 h at 37 °C) and washing with PBS-Tween 20 and a final wash with PBS. The steps to follow were the same as previously described.

Results and Discussion

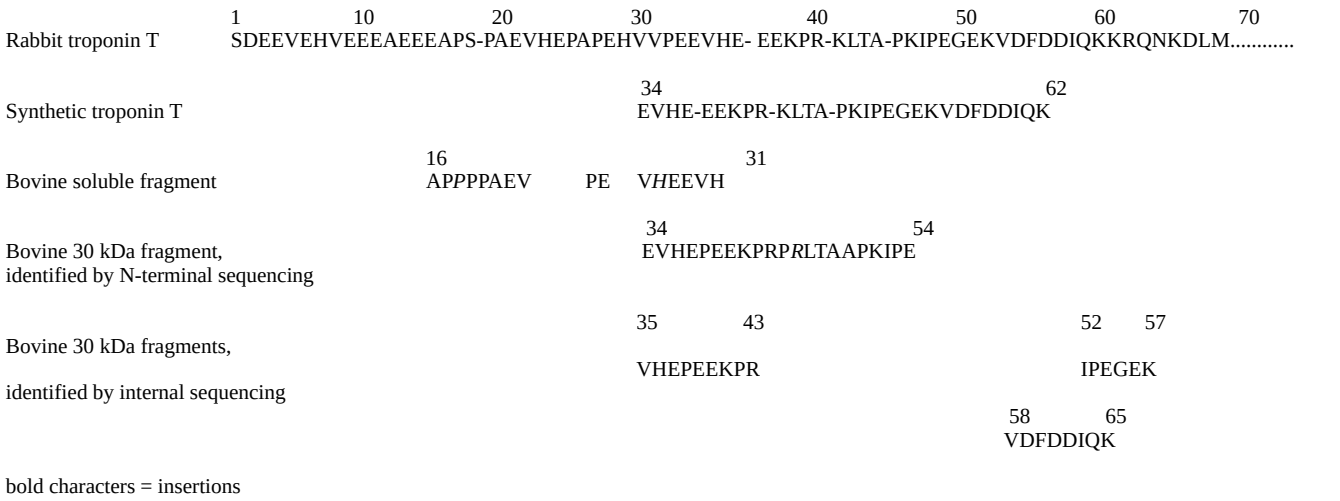
Elisa Development against the N-Terminus of the 30 kDa Fragment

Antigenic Site Predictions for the Troponin-T 30 kDa Fragment

Besides selected bovine troponin-T fragments, determined by internal microsequencing [11], we succeeded in the meantime to sequence the N-terminus of the 30 kDa fragment by insertion of the corresponding PVDF-blotted membrane pieces into the slot of the sequencer cartridge (see Materials and Methods), and the results of the first 21 Edman degradation cycles could be evaluated unequivocally, yielding as primary sequence EVHEPEEKPRPRLTAAPKIPE (Scheme 1). Aligning the 30 kDa fragments, identified by N-terminal as well as internal sequencing (Scheme 1), the primary structure of a N-terminal 29 amino acid residue troponin-T fragment could be identified, showing almost 90% homology to the known rabbit skeletal muscle troponin-T sequence. Based on this result, specific antibodies with a synthetic fragment could be produced, suitable for immunostaining experiments and development of an enzyme-linked immunosorbent assay (ELISA). One prerequisite for the formation of sensitive antibodies is, however, the synthetic fragment should provide a segment with a high antigenic index [24]. Immunostaining with specific antibodies against the 30 kDa fragment would allow us to discover changes in the skeletal muscle tissue, caused by modification of the troponin-T structure. A specific and sensitive ELISA would allow rapid, quantitative determination of troponin-T and its 30 kDa fragment in meat extracts for correlation studies with meat tenderness. This type of test would provide many advantages over the current more cumbersome SDS-PAGE method.

Before starting with the synthesis of the troponin-T fragment, we calculated the antigenic index, based on its primary sequence. Hydrophilic peptide segments, calculated according to [31], fragments with high side chain solvent accessibility, predicted according to [32], and highly flexible regions, calculated from X-ray diffraction-derived B factors [33], are known to be potential antigenic and immunogenic sites. However, as none of these programs has the ability to predict accurately enough the localization of antigenic sites in proteins, [23] developed a computer program to calculate potential antigenic determinants directly from the primary amino acid sequence of polypeptides and proteins integrating hydrophilicity (H), surface probability (S), back bone flexibility (F) and secondary structure (CF: Chou-Fasman [34]; GR: Garnier-Robson [35]) parameters, based on the following equation:

$$A_i = \sum_{i=1}^{N} 0.3\,(H_i) + 0.15\,(S_i) + 0.15\,(F_i) + 0.2\,(CF_i) + 0.2\,(GR_i)$$



Scheme 1 Comparison of the N-terminal primary structure of rabbit skeletal muscle troponin-T with bovine fragments, identified by sequence analysis

whereby $CF_i = GR_i = 2$ for strong turns, $CF_i = GR_i = 1$ for weak turns or random coil structures and $CF_i = GR_i = 0$ for α-helix or β-sheet segments.

Based on a computer plot of the total rabbit troponin-T sequence (data not shown), the segment 34–62 (Scheme 1) with the following primary structure was selected: $^{1(34)}$EVHE-EEKPR^{10}KLTAPKIPEG^{20} EKVD FDDIQ$^{29(62)}$K. A survey of the hydrophilicity (H) surface probability (S), flexibility (F), antigenic index (Ai) and secondary structure plots is shown in Figure 1. Highest antigenic index values are found around residues 8, 9 and 17, 18, based on positive values for the hydrophilicity, surface probablility, flexibility and β-turn parameters at these positions. Figure 2 shows a Chou-Fasman plot of the troponin-T segment 34–62 (1–29) with marked (by octagons) regions of a high antigenic index. The plot predicts an α-helical segment at the N-terminus, two β-turns (positions 8, 9 and 17, 18) and otherwise random coil structure for the 29 amino acid residue peptide.

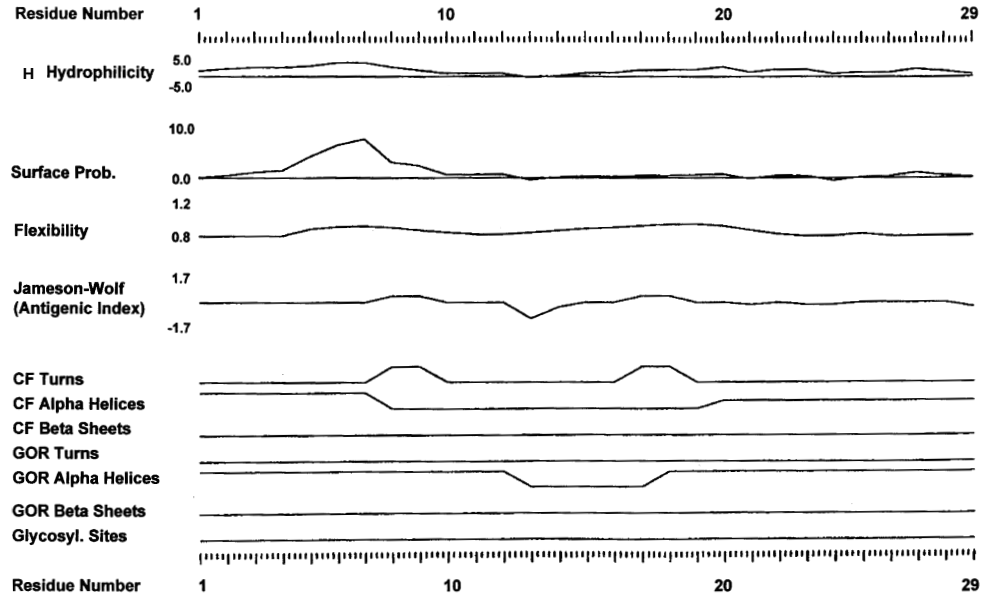


Fig. 1 Hydrophilicity (H), surface probability (S), flexibility (F), antigenic index (Ai), Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) plots, generated from the troponin-T fragment 34-62 (1-29) of the following primary structure: $^{1(34)}$EVHEEEKPR^{10}KLTAPKIPEG^{20} E KVDFDDIQ$^{29(62)}$K

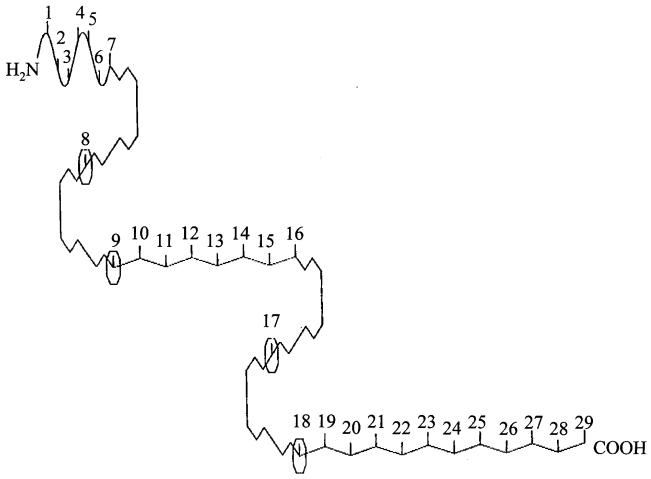


Fig. 2 Chou-Fasman secondary structure plot of the troponin-T fragment 34-62 of the following primary structure: $^{1(34)}$EVHEEEKPR^{10}KLTAPKIPEG^{20}EKVDFDDIQ$^{29(62)}$K. High antigenic index segments are marked by octagons; ∪: α-helix, ⌒: β-turn, ∨: random coil structure

Synthesis of Troponin-T Fragment **34–62**

According to the results received from the antigenic index plots (Figures 1, 2), the troponin-T segment 34–62 should be indeed a peptide fragment, suitable for the development of specific antibodies, recognizing the selected segment as well as the parent protein, as we could demonstrate in the past in connection with the development of specific immunoassays for quantitative determinations of thymosin β_9 [36], thymosin β_4 [37, 38], transmembrane glycoprotein gp41 env [39], human relaxin [40] or immunostaining experiments of β-thymosins [41, 25].

In our laboratory, solution [42], as well as solid phase [43–45, 26] peptide syntheses are well established for many years, and improved, highly efficient strategies are available. The advantage of solid phase compared to the solution method is that the side chain-protected peptide can be synthesized automatically on a solid support and no intermediate, but only final step purification of

the desired product is necessary. If mg to g quantities of the desired peptide are sufficient, as it is the case for antibody production, solid phase synthesis is the most rapid approach to produce the desired target molecule. Applying our experience over the last decade in peptide chemistry, the troponin-T 34–62 sequence was synthesized as follows: The most convenient strategy is to protect the α-amino functions temporarily by the Fmoc group, cleavable during the synthesis under mild basic conditions, *e.g.* 25% piperidine in DMF. Under these conditions, the side chain protecting groups (*tert*-butyl (Bu-*t*) for Asp, Glu, Thr, trityl (Trt) for His, Gln, *tert*-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl (Pmc) for Arg) are not cleaved and removed after completion of the synthesis with TFA, advantageously in the presence of scavangers. With this step, the assembled peptide is simultaneously cleaved from the solid support. Scheme 2 shows the structure of the protected troponin-T fragment 34–62, attached to the *p*-benzyl-oxybenzyl alcohol polystyrene resin. After cleavage of the deprotected peptide from the resin, the crude material was purified by gel chromatography and reversed-phase HPLC, as described under Materials and Methods. The high purity of the final product is demonstrated by the HPLC profile and LDMS spectrum, shown in Figures 3 and 4, respectively.

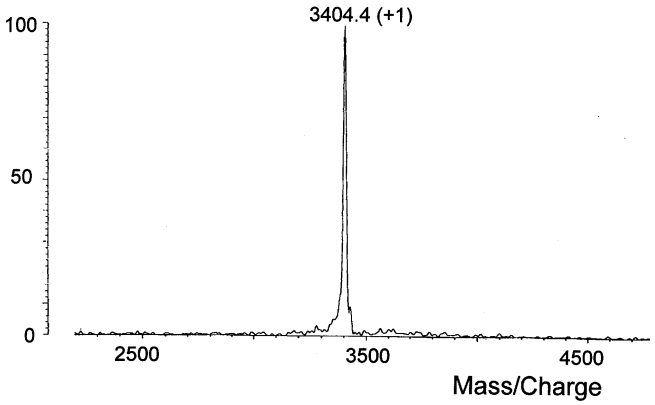


Fig. 4 MALDI spectrum of purified synthetic troponin-T 34–62 peptide fragment. Wavelength: 337 nm. Matrix: α-cyano-4-hydroxycinnamic acid

1 5 10
Fmoc - Glu(OBu^t) - Val - His(Trt) - Glu(OBu^t) - Glu(OBu^t) - Glu(OBu^t) - Lys(Boc) - Pro - Arg(Pmc) - Lys(Boc) - Leu - Thr(OBu^t) - Ala - Pro -

15 20 25 29
Lys(Boc) - Ile - Pro - Glu(OBu^t) - Gly -Glu(OBu^t) - Lys(Boc) - Val - Asp(OBu^t) - Phe - Asp(OBu^t) - Asp(OBu^t) - Ile - Gln(Trt) - Lys(Boc) - resin

Scheme 2 **S**tructure of the protected troponin-T 34–62 segment, synthesized on a *p*-benzyloxybenzyl alcohol polystyrene resin (Bu^t = *tert*-butyl, Trt = trityl, Boc = *tert*-butyloxycarbonyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl)

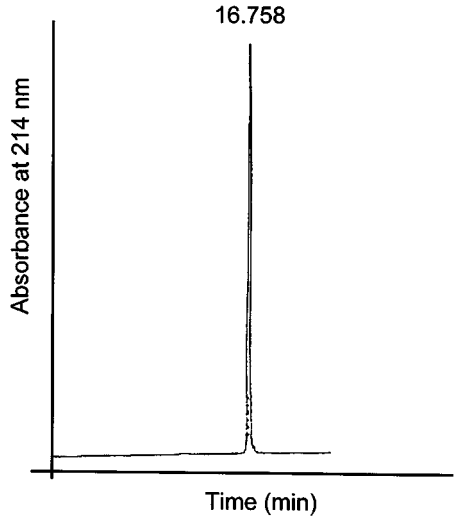


Fig. 3 HPLC profile of purified synthetic troponin-T 34–62 peptide fragment. Column: Nucleosil 100 C_{18} (250 × 20 mm, 7 µm; Macherey-Nagel, Düren, Germany). Gradient: 5–90% B in 31 min; A: 0.05% TFA in H_2O; B: 60% CH_3CN, 0.08% TFA in H_2O. Flow rate: 1mL/min. Detection: UV at λ = 214 nm

Specificity of Antibodies, Generated against the Synthetic TNT 34–62 Fragment

The antibodies generated against the synthetic TNT 34–62 fragment, spanning the amino acid sequence of rabbit TNT 34–62, were tested in ELISA an. Using the first protocol (protocol I in Materials and Methods), the useful range of the assay, as obtained from the standard curve, was 10–100 ng of TNT 34–62 (Figure 5).

A similar standard curve was obtained using protocol II, as described in Materials and Methods. Concerning the specificity of the generated antisera, four known standard proteins (BSA, ovalbumin, ribonuclease and aprotinin) were tested and no cross-reaction with any of them was observed, even at high concentrations of 5 µg/mL (data not shown). In a first series of meat sample preparations, extracts containing SDS were analyzed, however, strong disturbance of the assay was observed due to the presence of the detergent. As Tris-buffered

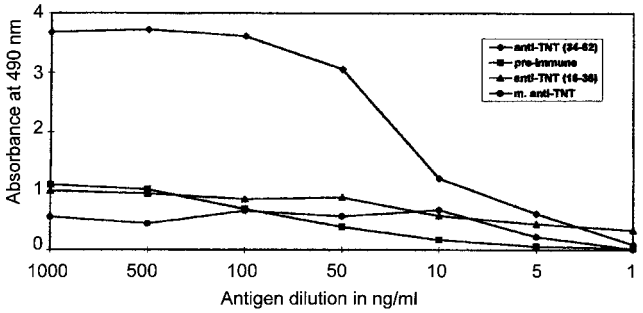


Fig. 5 Standard curve of troponin-T 34–62. EIA plates were coated with 1 ng to 1 µg of peptide. Anti-troponin-T 34–62, and pre-immune serum were used at a dilution of 1:10 000. Monoclonal anti-troponin-T (m. anti-TNT; Sigma) was used at 1:200. Assays were performed as described in Material and Methods

saline (TBS) is used as a reagent in both ELISA procedures (protocol I and II), TBS-extracted meat samples were prepared and tested (for experimental details see Materials and Methods). Three different bovine MLDs (0.2 g), after storage at 24 h, 7 d and 21 d were homogenized in 20 ml TBS buffer and the supernatants analyzed by the ELISA. In all three samples the highest amount of cross-reactive material as expressed in troponin-T 34–62 equivalents was detected after 24 h storage (average value of 23 ng/mL), decreasing 4- to 8-fold after storage for 7 days and 21 days, respectively. The antibodies generated against the N-terminus of the 30 kDa troponin-T 34–62 fragment, should cross-react with the synthetic sequence (as proven by the standard curve), with the 30 kDa troponin-T protein, with troponin-T itself and also with fragments containing antigenic sites of the synthetic sequence. The decrease in cross-reactive material with storage time of the TBS-extracted meat samples is in contrast to the increased appearance of the 30 kDa band. This observation can be explained possibly by the presence of protein fragments with troponin-T 34–62 segments which are degraded after longer storage. However, further systematic studies are needed to correlate these ELISA values with parameters of meat quality.

Meat Quality Markers from Soluble Meat Extracts

SDS-PAGE profiles of sarcoplasmic proteins are commonly used for evaluation of meat tenderness. However, this operation is tedious, inconvenient, time-consuming and not suitable for automation. For routine analysis of larger numbers of samples, HPLC, CE and immunoassay procedures are much better suited, as fully automated systems are already commercially available. However, these methods yield reliable results only, if meat quality indicators are provided in solution. Therefore, we concentrated in the last months on analyzing supernatants of TCA- and HCl-treated samples concerning changes of free amino acids and oligopeptides during beef storage [46, 47].

Investigations on Soluble TCA Meat Extracts

HPLC Profiles of Soluble TCA Meat Extracts

From 3 Hereford cross heifers, slaughtered, hung conceptionally at 1 h *post-mortem*, the M. *longissimus dorsi* (MLD) were excised, stored at 4 °C and samples taken from each of 8 different locations along the muscles at 1 h, 1 d, 3 d and 15 d, treated with 5% TCA and the components of the supernatants further separated by HPLC. Besides low molecular weight components, eluted at low retention time, 4 prominent peaks are observed in the chromatograms, increasing with muscle ageing, and, therefore we suggest these components to be useful indicators of meat quality [48]. For demonstration, Figure 6 shows the HPLC profiles of TCA-soluble extracts of a beef sample after storage for 3 and 15 days.

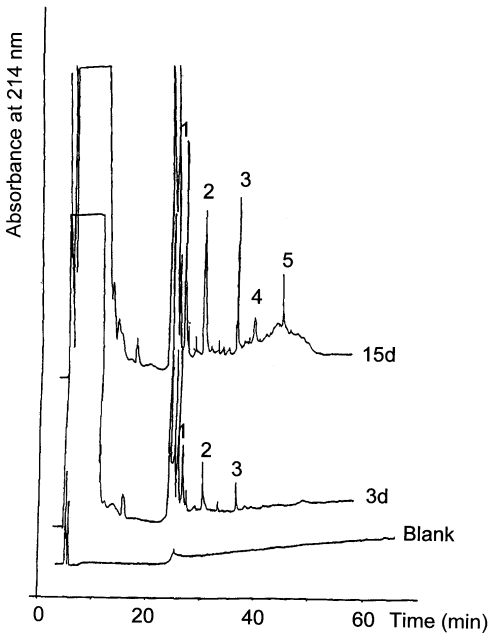


Fig. 6 HPLC profiles of TCA-soluble extracts of beef samples after storage for 3 and 15 days (d), respectively. 400 µL of the extracts (mainly salts, free amino acids and peptides) were injected directly into the Rheodyne valve. Column: LiChrospher RP-18 (250 × 4 mm; 5µm; Merck, Darmstadt). Gradient: 0 to 70% B in 60 min; A: 0.1% TFA in H_2O; B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1.0 mL/min. Detection: UV at λ = 214 nm

Structure Determination of Prominent Soluble Components of TCA Meat Extracts

After collection of fractions 1–5, lyophilisation (Figure 6), sequencing and LDMS analysis, fractions 1 and 2 were found to be free amino acids (Tyr and Phe, respectively). In fraction 3, a fragment of glyceraldehyde-3-phosphate dehydrogenase (KVVKQASEGPLK

(12AA)), in fraction 4, a fragment of troponin-T (APPPPAEVPEVHEEVH (16AA)) and in fraction 5, a fragment of creatine kinase (DPIIQDRHEGFKPTDKHKTDLNHENLKGGDDLDPNYVLS (39AA)) with chains lengths of 12, 16 and 39 amino acid residues, respectively, could be identified.

From supernatants of TCA-treated beef samples, [49] a troponin-T segment was isolated by HPLC and identified which is in coincidence with the structure of our fraction 4 except for the C-terminal histidine residue [47], overlooked by the Japanese laboratory, and eventually of importance for development of specific antibodies. Comparison of the overlapping sequences of the C-terminus of the soluble bovine troponin-T fragment with the N-terminus of the 30 kDa troponin-T fragment (Figure 1) furthermore confirms our results.

As mentioned in the introduction, quite a number of changes have been observed during post-mortem storage of meat, some of which could be correlated with meat tenderness. One of the most noticeable is the disappearance of the TNT and a simultaneous appearance of a 30 kDa band as observed by SDS-PAGE [2, 3, 4, 11, 47, 49, and references quoted therein]. As described in the beginning of this communication, we unequivocally determined the 30 kDa band to be a fragment of TNT *via* Edman degradation of the electroblotted protein band. Surprisingly, we could identify in TCA-soluble meat extracts another TNT fragment (16–31), the quantity of which is increasing with storage of meat (Figure 6, fration 4), as also observed in a similar way for the intensities of the SDS-PAGE spots of the 30 kDa band (data not shown). For quantitative determination and correlation with meat quality *e.g. via* HPLC, CE or immunoassays, the soluble TNT fragment is far better suited than the insoluble 30 kDa fragment, and therefore specific antibodies were developed, as descibed in the following section. Besides TNT (16–31), soluble fragments of glyceraldehde-3-phosphate dehydrogenase and creatine kinase were identified, also in increasing quantities with muscle ageing; their appearance and correlation with meat quality is a matter of further investigations.

Synthesis of Troponin-T Fragment 16–31

Sequence comparison of the bovine TNT 16–31 peptide segment, isolated as fraction 4 (Figure 6) from TCA meat extracts, with that of rabbit troponin-T reveals (Scheme 1) that its N-terminal alanyl–prolyl residue is identical with position 16 and 17 of the rabbit protein. Furthermore, it overlaps with its 3 C-terminal amino acid residues with the N-terminus of the 30 kDa fragment, identified by N-terminnal sequencing [11]. As the concentration of the soluble TNT 16–31 fragment in the TCA extracts seems to be a potential marker for meat quality, development of an ELISA for its routine determinations would be of interest. From the antigenic index plot, calculated from hydrophilicity, surface probability and flexibility parameters, it becomes obvious that throughout its sequence positive values are predicted (Figure 7).

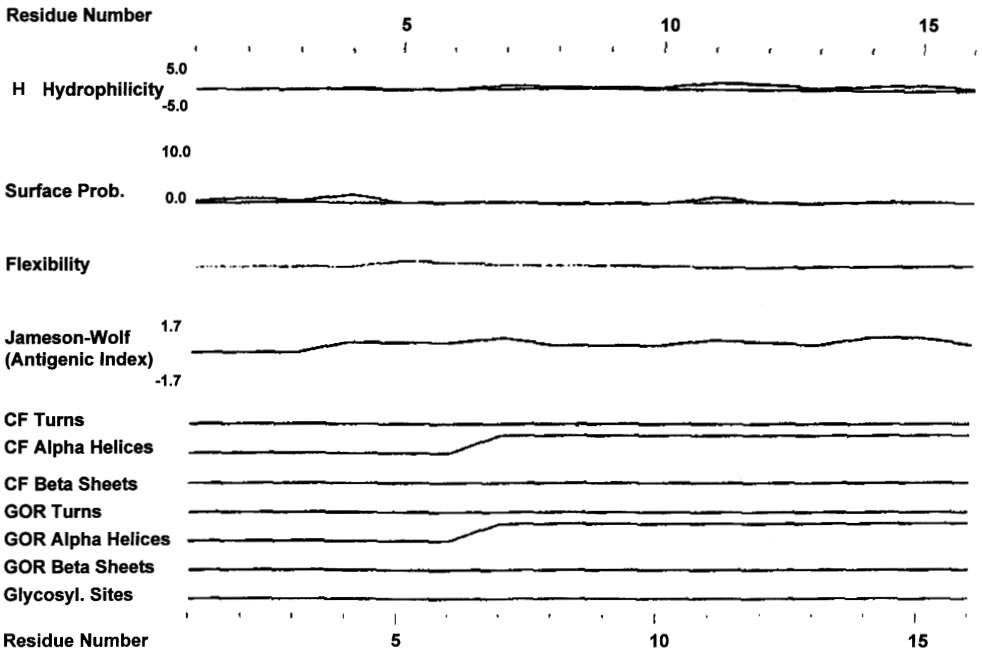


Fig. 7 Hydrophilicity (H), surface probability (S), flexibility (F), antigenic index (Ai), Chou-Fasman (CF) and Garnier-Robson (GOR) secondary structure plots, generated from the solouble troponin-T fragment 16–31 (1–16) of the following primary structure: A(1(16))PPPPAE(10)VPEVHEEVH(16(31)

1 5 10
Fmoc - Ala - Pro - Pro - Pro - Pro - Ala - Glu(OBu^t) - Val - Pro - Glu(OBu^t) - Val -

16
His(Trt) - Glu(OBu^t) - Glu(OBu^t) - Val - His(Trt) - resin

Scheme 3 Structure of the protected troponin-T 16–31 (1–16) segment, isolated from meat TCA extracts and synthesized on a *p*-benzyloxybenzyl alcohol polystyrene resin (Bu^t = *tert*-butyl, Trt = trityl).

Therefore, the peptide was synthesized according to the Fmoc strategy as discussed above, using side chain protecting groups as given in Scheme 3. The high purity of the final product is demonstrated by a single HPLC fraction (Figure 8) and its LDMS-spectrum, showing one peak at *m/z* 1731.2 [MH$^+$]. The purified peptide was used for antibody development as described in Materials and Methods.

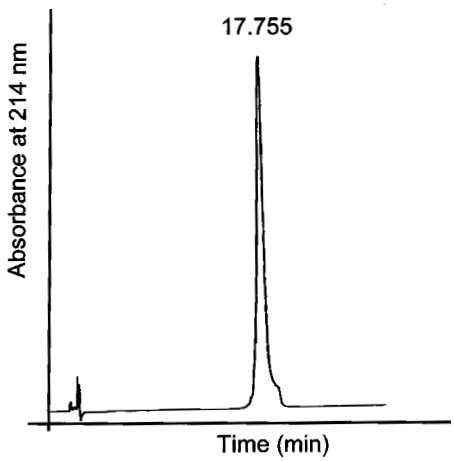


Fig. 8 HPLC profile of purified synthetic troponin-T 16–31 (1–16) fragment, isolated from TCA meet extracts. Column: Nucleosil 100 C_{18} (250 × 20 mm, 5 µm; Macherey-Nagel, Düren, Germany). Gradient: 5–90%B in 36 min; A: 0.35% TFA in H_2O; B: 60% CH_3CN, 0.29% TFA in H_2O. Flow rate: 1mL/min. Detection: UV at λ = 214 nm

ELISA Development against the Soluble Troponin-T 16–31 Fragment

Polyclonal antibodies generated *via* rabbit immunizations against synthetic troponin-T 16–31 were used for ELISA development. This assay presented high specificity for the TNT 16–31 peptide with a useful range of 5–50 ng troponin-T 16–31 equivalents/mL (Fig. 9). No cross-reaction with high concentrations of known proteins (data not shown) was observed. Additionally, no cross-reaction was found with the troponin-T 34–62 peptide, demonstrating that these antibodies do not recognize the soluble troponin-T 16–31 fragment though the overlapping EVH tripeptide at the N-terminus and C-terminus of the molecules, respectively. This, as expected, holds true in the opposite direction too, *i.e.*, the

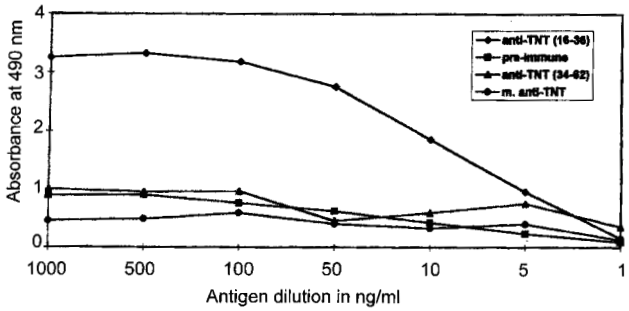


Fig. 9 Standard curve of troponin-T 16–31. Anti-troponin T 16–36 was used at a dilution of 1:10 000. Further details see

produced antibodies against troponin T 34–62 do not recognize the troponin-T 16–31 sequence (Fig. 5). Using this assay, 3 fractions (marked peaks 3, 4 and 5; Figure 6) isolated after HPLC analysis of the soluble extract were analyzed. As expected and in coincidence with the data available through protein sequence analysis, cross-reactive material was detected only in fraction 4, although the levels measured were low (7 ng troponin-T 16–31 equiv./mL), possibly due to the low concentration of the soluble material contained in the sample. Analysis of TCA extracts revealed higher levels (by 2-fold) of cross-reactive material on day 15 as compared to day 3, whereas marginal levels were detected in 1-day and 3-hours samples. The HCl extract analysed (see below) contained no troponin-T soluble fragment and TBS extract analysis is in progress. It should be pointed out that the main advantage of such samples in our assay is the use of volatile agents like TCA or HCl for the muscle extract preparation and the complete lack of SDS in the whole process, the latter routinely used in the preparation of myofibrilar proteins and whose presence in an immunoassay is strictly prohibited.

Capillary Electrophoresis of Soluble TCA Meat Extracts

Minimal sample and buffer requirements in combination with rapid and efficient separation made capillary electrophoresis (CE) one of the most attractive tools for the analysis of biopolymers like peptides, proteins, glycoproteins or oligonucleotides, as several selected references demonstrate [50–53, 22]. Very recently, pharmaceutical and clinical analysis laboratories started to develop routine CE methods for purity testing, quantitative determinations of the formulation content, chiral

analysis, monitoring of drugs in body fluids or reliable and precise analysis of blood. The wide application and advantages of CE over other analytical tools should be attractive also for meat scientists to develop routine methods for the quantification of meat quality markers based on capillary electrophoretic separation, as demonstrated here by just a few preliminary examples.

In capillary zone electrophoresis (CZE), the most commonly applied separation mode in CE, the solutes are separated in a single solution buffer. The ratio of charge to size of the analytes is the basis of separation, whereas the choice of the pH of the running buffer plays a crucial role, because it influences the charge of the sample component. Normally, CZE is performed in bare fused-silica capillaries, but for protein separations, for example, it is recommended that coated capillaries or buffer additives be used to suppress adsorptions to the capillary wall. To demonstrate the advantages of CE over HPLC, separations with both methods of identical soluble meat extracts are compared with each other.

For structure determination, including Edman sequencing, fractions 1 to 5 of TCA meat extracts (Figure 8) were pooled and rechromatographed. The HPL chromatograms of pooled fraction 3 (glyceraldehyde-3-phosphate dehydrogenase 258–269 fragment) and 4 (troponin-T 16–31 fragment) are shown in Figures 10 and 11, and for comparison, the capillary electropherograms of the same samples are drawn in Figures 12 and 13, respectively. Under the chromatographic conditions, as stated in the legends of Figures 10 and 11, the main components of both fractions are eluted after about 30 min,

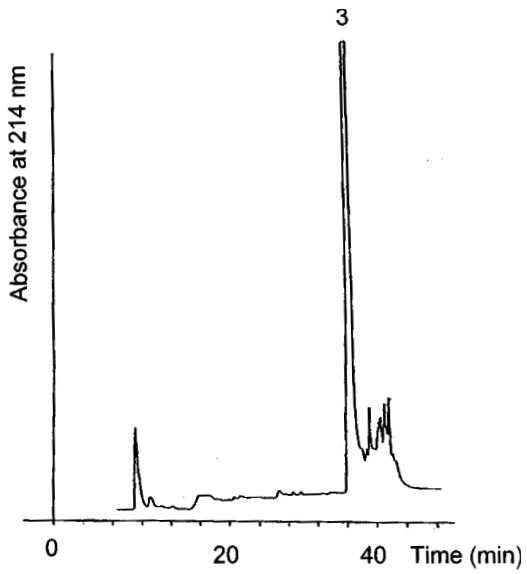


Fig. 10 HPLC profile of pooled fraction 3 (Figure 6) of TCA meat extracts. Column: LiChrospher RP-18 (250 × 4 mm, 5µm; Merck, Darmstadt). Gradient: 0–45% B in 60 min; A: 0.1% TFA in H_2O, B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1mL/min. Injection volume: 100 µL. Detection: UV at λ = 214 nm

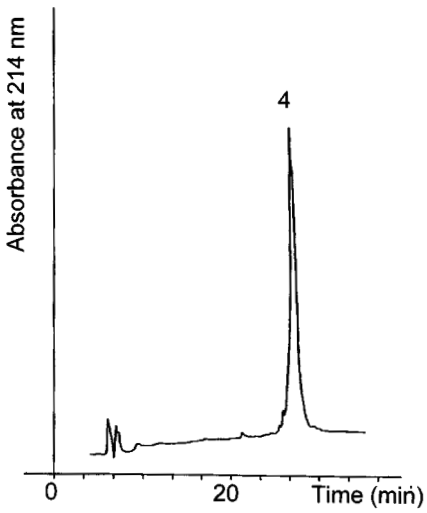


Fig. 11 HPLC profile of pooled fraction 4 (Figure 6) of TCA meat extracts. Column: LiChrospher RP-18 (250 × 4 mm, 5µm; Merck). Gradient: 5–60% B in 60 min; A: 0.1% TFA in H_2O, B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1mL/min. Injection volume: 100 µL. Detection: UV at λ = 214 nm

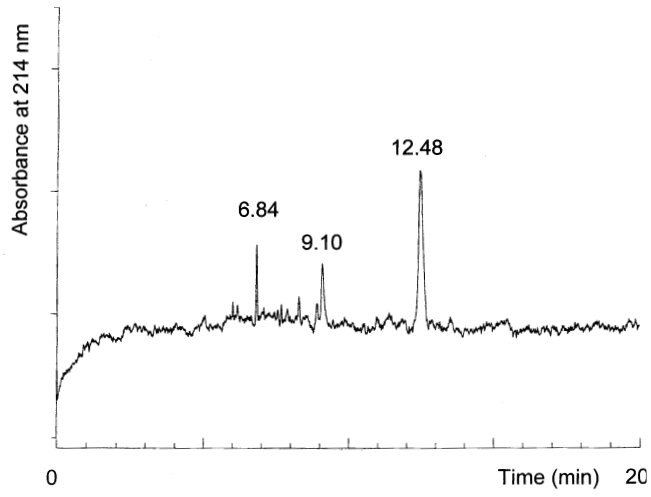


Fig. 12 Capillary electropherogram of pooled fraction 3 (Figure 6; Figure 10) of TCA meat extracts. Capillary: Uncoated fused silica (55 cm × 50 µm i.d.; Grom, Herrenberg, Germany). Pressure Injection: 10 psi × s. Running conditions: 20 kV. Capillary temperature: 20 °C. Buffer: 50 mM phosphate buffer, pH 2.5. Polarity: Anode at the inlet. Detection: UV at λ = 195 nm

and HPLC separation allows to detect minor amounts of side products in fraction 3 (Figure 10), but no contaminations for fraction 4 (Figure 11). The superiority of CE over HPLC separation is clearly demonstrated from the electropherograms shown in Figures 12 and 13: The main components of fraction 3 and 4 are eluted in much shorter time (10–12 min), contaminations can be far better detected, nanoliter amounts are only needed for the separation, and migration times of peptides can be predicted from their net charge and molecular

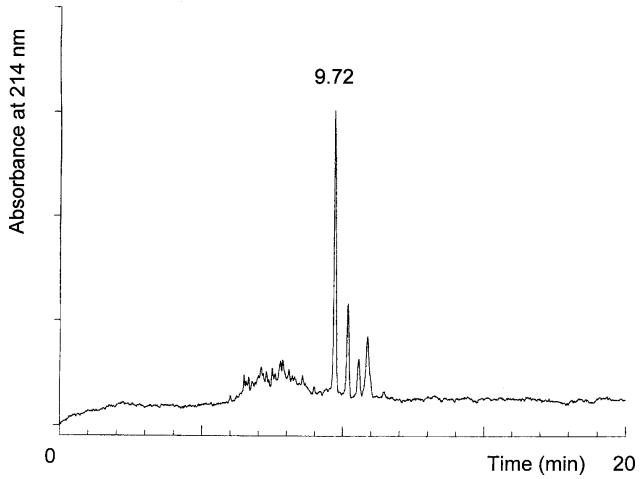


Fig. 13 Capillary electropherogram of pooled fraction 4 (Figure 6; Figure 11) of TCA meat extracts. Experimental conditions: See Figure 12

mass. Therefore, due to rapid separation (3 to 10 min after optimization), high resolution (up to several million theoretical plates) and high sensitivity (attomole range), capillary electrophoresis may become an attractive candidate for routine analysis of meat quality markers in full automatic mode.

Preliminary Results on Soluble HCl Meat Extracts

In order to find the most rapid, convenient and cheapest work up precedures preparing soluble meat extracts, suitable for analyzing meat quality markers, we prepared also HCl extracts (see Experimental) and started identifying their components. As the comparison of the HPL chromatograms of soluble meat extracts, collected after TCA (Figure 6) and HCl (Figure 14) extraction, respectively, shows that there is a dramatic difference in the profiles, depending on the treatment used. Compared to the TCA extracts, exhibiting 6 prominent peaks in the range of 20–60 min retention time, the chromatogram of the HCl extract shows tiny peaks within this time period, but instead, a broad one between 60 and 80 min, caused by column overloading. After collection of this unresolved fraction and rechromatography (Figure 15), 5 proteins could be separated, lyophilized and Edman degradation yielded the N-terminal sequences of the following proteins according to our search for protein homologies: ^{1}PHQYPALTP^{10} EQKKE^{15}L (peak 1, Figure 15), reveals 100% identity with fructose-biphosphate aldolase from frog (positions 1–15), ^{1}SFGNTHNKX^{10} KLNFKAEEEY^{20}P (Peak 2, Figure 15) is 95% identical in 20 overlapping amino acid residues with a fragment of creatine kinase 1–20 of rabbit, mouse and human, ^{1}VKVGVNGFG^{10}RIGRLVTRAA^{20}F (Peak 3, Figure 15) shows 100% identity with the N-terminal sequence of glyceraldehyde-3-phosphate dehydrogenase

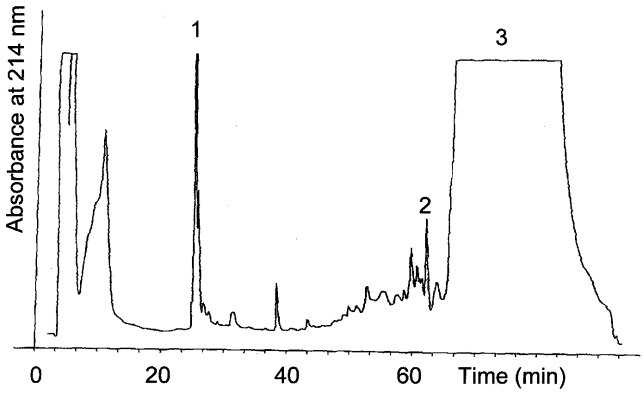


Fig. 14 HPLC profile of HCl-soluble extract of a beef sample after storage for 3 days. 150µL of the extract were diluted injected into the Rhodyne valve. Column: LiChrospher RP-18 (250 × 4 mm, 5µm; Merck). Gradient: 0 to 70% B in 60 min; A: 0.1% TFA in H_2O, B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1.0 mL/min. Detection: UV at λ = 214 nm

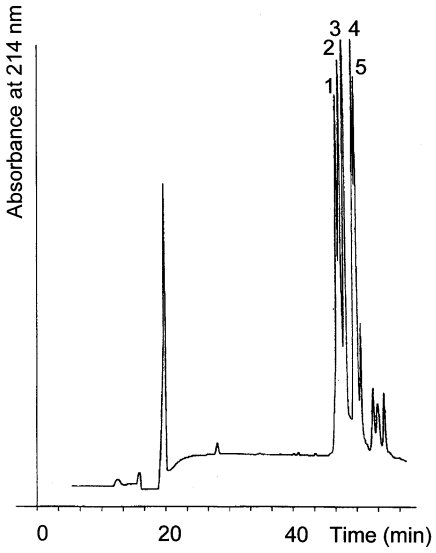


Fig. 15 HPLC profile of HCl-soluble extract of a beef sample after storage for 3 days. 250 µg/ 250µL (Fraction 3, see Figure 14) were injected into the Rhodyne valve. Column: Nucleosil 100 RP-18 (250 × 10 mm; 7 µm; Macherey-Nagel, Düren, Germany). Gradient: 15 to 100% B in 60 min; A: 0.1% TFA in H_2O, B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1.0 mL/min. Detection: UV at λ = 214 nm (Concerning numbering of the peaks, see text)

from rat, pig and human and ^{1}GLSDGEWQL^{10} VLNAWGKVE^{19}A (Peak 4 and 5, Figure 15) is found to be 100% identical in 19 overlapping amino residues with the N-terminal fragment of bovine myoglobin. It is worthwhile mentioning that the ELISA developed against the troponin-T 16–31 segment does not show any cross-reactivity, with the soluble HCl meat extract (see above).

These proteins, found in the soluble HCl meat extract could be further candidates to serve as markers for meat

quality, however, so far no correlations between time-related quantitative appearance of these proteins and meat conditioning have been made. As demonstrated for the soluble TCA meat extracts, HPLC, CE or immunoassay methods could be developed for specific quantitative routine analysis of these potential markers of meat quality in soluble HCl extracts.

Conclusions

According to several reports in the literature, the disappearance of the TNT and the appearance of a 30 kDa band in SDS-PAGE of myofibrillar extracts from bovine meat, seem to be good indicators of the extent of post-mortem meat proteolysis and are also correlated with meat tenderness [4]. In the present report unequivocal proof is given for the origin of the 30 kDa band by protein sequence determination to be a fragment of TNT. As SDS-PAGE is a tedious and besides inaccurate procedure for quantitative determination of proteins, soluble TCA and HCl meat extracts were analysed and selected protein/peptide components identified by sequencing. One peptide fraction in the TCA-soluble meat extract is identical to the TNT (16–31) sequence and its concentration increases with muscle ageing in a similar way as the 30 kDa band in SDS-PAGE and can therefore be also considered to be a good indicator of the extent of post-mortem meat proteolysis. As this peptide marker is a component of soluble extracts, HPLC, CE or immunoassay methods are equally suitable for its rapid quantitative routine analysis, what is demonstrated by the different elaborated methods. Besides the TNT fragment, several other peptides/proteins were identified by isolation and sequencing from TCA- and HCl-soluble meat extracts; their quantity increases also with the time of muscle storage, but their potential as meat quality markers has to be evaluated by further studies.

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