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Determination of changes in protein conformation caused by pH and temperature

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Abstract

Protein denaturation has a major impact on meat quality parameters such as water holding capacity, tenderness and color. Specific information about structural changes of the individual muscle proteins post-mortem could help understand the factors affecting meat quality. An aromatic dye, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bisANS) that binds to the hydrophobic patches of proteins was used to monitor changes in the conformation of individual sarcoplasmic proteins caused by pH. The bisANS reagent was covalently linked to the proteins with UV-light and the proteins were separated and identified using gel electrophoresis and mass spectrometry. The results showed that the sarcoplasmic proteins creatine kinase M, aldolase A and lactate dehydrogenase showed increased hydrophobicity whereas carbonic anhydrase III showed decreased hydrophobicity with increasing pH. Temperature only had a marked effect on the results at around 40 °C, there being no change between 25 and 35 °C.

Keywords: pH; BisANS; Sarcoplasmic proteins; Hydrophobic protein domains

1. Introduction

Protein denaturation has an impact on such meat quality parameters as water holding capacity and color (Offer & Night, 1988; Sayd et al., 2006). It is well established that protein denaturation is in part responsible for the quality alterations observed in PSE meat (Warner, Kauffman, & Greaser, 1997). Furthermore, the rate and extent of the pH decline post-mortem are of major importance to ultimate meat quality and it has also been shown that post-mortem metabolism may be affected by denaturation of metabolic proteins (Ryu, Choi, & Kim, 2005).

Different methods have been used to study denaturation of proteins post-mortem such as quantification of myofibrillar ATPase activity, protein solubility and changes in protein hydrophobicity (Mignino & Paredi, 2006; Ryu et al., 2005; Warner et al., 1997; Yongsawatdigul & Park, 2003). The most used method for monitoring protein denaturation post-mortem is probably the quantification of the myofibrillar ATPase activity which is relatively easy and effective. However, with this method, only the denaturation of a single protein is estimated. Studies have used changes in protein hydrophobicity to estimate protein denaturation, using different hydrophobic probes such as bromophenol blue (Chelh, Gatellier, & Sante-Lhoutellier, 2006) and 8-anilino-1-naphthalenesulfonic acid (ANS) (Mignino & Paredi, 2006; Yongsawatdigul & Park, 2003). In these studies, an estimation of total protein denaturation is obtained but it is not possible to estimate the denaturation of individual proteins.

The aromatic dye 4,4′-dianilino-1,1′-dinaphthyl-5,5′-disulfonic acid (bisANS) is capable of recognizing the hydrophobic patches on proteins (Fu, Zhang, & Chang, 2005). The interactions between bisANS and proteins are non-covalent and reversible. However, it was shown that these interactions could become covalent after irradiation

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of protein-bisANS with longwave UV-light, thus providing more specific information of the individual proteins (Seale, Martinez, & Horowitz, 1995). Recently, bisANS has been used to screen for single protein conformational changes in a complex protein mixture using 2D electrophoresis, demonstrating that this method can be used to screen a complex mixture of proteins for alterations in surface hydrophobic domains of individual proteins (Pierce, de Wall, Van Remmen, Richardson, & Chaudhuri, 2006). In the present study, we used bisANS to screen for conformational alterations in individual sarcoplasmic proteins caused by pH changes.

2. Materials and methods

2.1. Reagents

BisANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid), ammonium bicarbonate, Trizma HCl, Trizma base, 2-(*N*-morpholino) ethanesulfonic acid (MES), Coomassie brilliant blue G-250 and $MgSO_4$ were purchased from Sigma–Aldrich (Copenhagen, Denmark).

The bicinchonic acid assay (BCA) kit was obtained from Pierce (Hercules, CA). Complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics GmbH (Mannheim, Germany) and ethanol, acetonitrile and acetic acid were obtained from Bie & Berntsen (Rødovre, Denmark). NuPage MES Running buffer, NuPage LDS sample buffer and NuPage 4–12% Bis–Tris gel (100 × 15 well) were obtained from Invitrogen (Carlsbad, CA). Trypsin gold mass spectrometry grade was purchased from Promega (Madison, WI) and DL-1,4-dithiothreitol from ACROS organics (Geel, Belgium).

2.2. Sarcoplasmic extract

Five grams of pig *Longissimus dorsi* muscle (2 h after slaughter) were homogenized with an Ultra turrax Mixer T25 (Janke & Kunkel, Staufen, Germany) in six volumes of homogenization buffer (50 mM Tris, 10 mM $MgSO_4$ and protease inhibitors (one tablet for 50 ml buffer), pH 7.4).

Extracts were centrifuged for 30 min at 15,400*g* (4 °C) and the supernatant was filtered. Protein content was determined by the bicinchoninic acid (BCA) assay and extracts diluted to 1 mg/mL with the appropriate buffer MES 100 mM or Tris 100 mM at different pH's (Tris 7.5, 7.0 and MES 6.5, 6.0, 5.5, 5.0). Samples were finally incubated in a water bath for 30 min at 35 or 25 °C.

2.3. Photoincorporation of bisANS

The photoincorporation process of bisANS was carried out as described by Seale et al. (1995) with slight modifications. Four microliters of a 10 mM bisANS methanolic solution was added to 200 μl extract (1 mg/ml) followed by incubation on ice for 10 min. Afterwards, extracts were put on a 96-well micro plate and exposed to UV-light for 10 min with a UV-lamp (Model UVL-56, 366 nm, Ultraviolet Products Inc., Upland, CA). The UV-lamp was placed on top of the micro plate and the micro plate was put on ice to minimize overheating of samples.

2.4. Gel electrophoresis

The protein samples were separated by SDS–PAGE (200 V, 50 min), using NuPage 4–12% Bis–Tris Gels (15 well) and MES buffer as running buffer. The gels were equilibrated for 10 min with a mixture of ethanol:acetic acid:water (40:10:50) v/v/v and illuminated with the UV-light. The bisANS incorporation was quantified by excitation with UV-light and measuring the emission at 605 ± 20 nm with the Camilla II fluorescence camera system (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). Then, gels were stained with colloidal Coomassie. The quantification of bands stained by colloidal Comassie or bisANS was performed using the Phoretix Software (version 2003.02, Nonlinear Dynamics Ltd., Newcastle, UK).

2.5. Protein identification

Selected protein bands were cut out of the gels and digested with trypsin as described by Lametsch et al. (2006). The resulting peptides from the protein digestion were analyzed with the use of a matrix-assisted laser desorption ionization system time-of-flight mass spectrometer (MALDI-TOF-TOF) (Ultraflex II, Bruker Daltonics, Bremen, Germany). Both mass spectrometry (MS) and MS/MS data was used for protein identification. The Database search was performed using the Mascot database search program with a peptide mass tolerance of ±50 ppm and a fragment mass tolerance of ±0.5 kDa.

3. Results and discussion

Preliminary trials were carried out to determine the optimal conditions for covalent linking of bisANS to the proteins. UV irradiation is necessary for binding bisANS covalently to the proteins. However, UV irradiation also can cause the cross-linking of proteins so the UV irradiation has to be limited. It was found that 10 min was the optimal UV irradiation time, giving no detectable cross-linking of proteins.

The effect of pH and temperature on the hydrophobicity of individual sarcoplasmic proteins from pig *Longissimus dorsi* muscle was investigated.

The results clearly demonstrate that the amount of covalently bound bisANS to some of the sarcoplasmic protein changes with pH (Figs. 1 and 2), reflecting changes in the hydrophobicity of the proteins, presumably caused by protein denaturation. In all cases, no differences in band intensities of different samples stained with colloidal Coomassie were observed, illustrating that the changes in bisANS intensity were not caused by protein cross-linking since

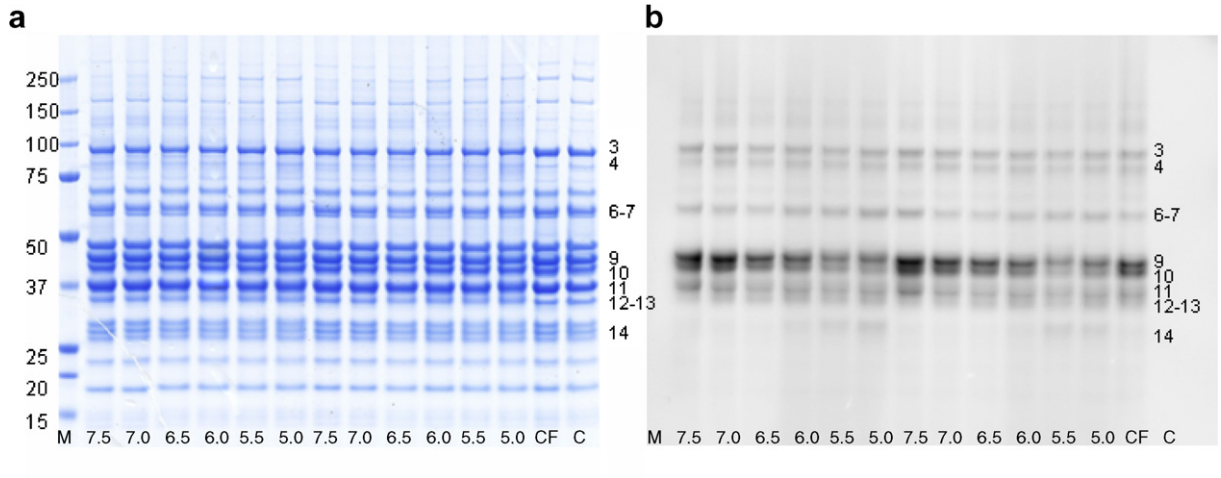

Fig. 1. (a) Colloidal Coomassie staining of the gel. Samples were loaded as follows: molecular weight marker (M), samples heated at 35 °C at different pH's (7.5, 7.0, 6.5, 6.0, 5.5, 5.0), duplicates of samples heated at 35 °C at different pH's (7.5, 7.0, 6.5, 6.0, 5.5, 5.0), control sample with bisANS and exposed to UV-light (CF) and control sample without bisANS and not exposed to UV-light (C). Protein (6.5 μg) loaded in each lane. (b) Fluorescence signal of bisANS (λ_{ex} UV and λ_{em} 605 nm) of the same gel as in (a).

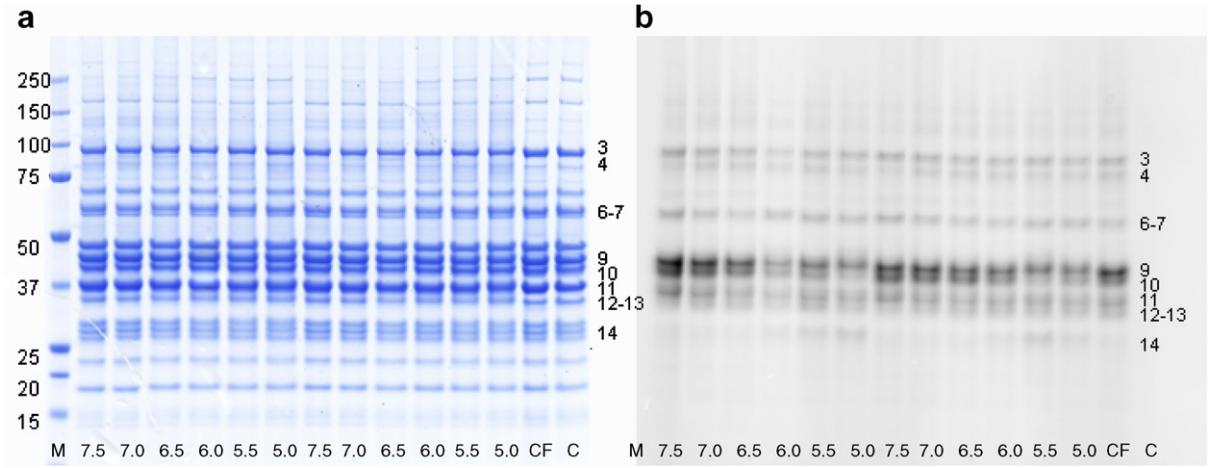

Fig. 2. (a) Colloidal Coomassie staining of the gel. Samples were loaded as follows: molecular weight marker (M), samples heated at 25 °C at different pH's (7.5, 7.0, 6.5, 6.0, 5.5, 5.0), duplicates of samples heated at 25 °C at different pH's (7.5, 7.0, 6.5, 6.0, 5.5, 5.0), control sample with bisANS and exposed to UV-light (CF) and control sample without bisANS and not exposed to UV-light (C). Protein (6.5 μg) loaded in each lane. (b) Fluorescence signal of bisANS (λ_{ex} UV and λ_{em} 605 nm) of the same gel as in (a).

protein cross-linking would change the molecular weight and modify migration on the gel. BisANS bound to some sarcoplasmic proteins; samples without bisANS showed no fluorescent signal.

Several of the protein bands from the SDS–PAGE gels where identified by mass spectrometry (Table 1). Some of the protein bands showed a decrease in the fluorescence intensity with decreasing pH (bands 9–11, Figs. 1 and 2) while another showed an increase (band 14, Figs. 1 and 2). However, the absolute quantity (Coomassie staining) was not affected.

The three protein bands gave a decrease in the fluorescence intensity with decreasing pH were identified as creatine kinase, fructose-bisphosphate aldolase A (muscle-type aldolase) and dehydrogenase glyceroaldehydephosophatase. The protein band found to increase in fluorescence intensity with decreasing pH was carbonic anhydrase III (Fig. 3). All of these proteins are metabolic enzymes that play an important role in the maintenance of cellular homeostasis.

Aldolase A is an enzyme that catalyses one of the aldol reactions: The substrate fructose 1,6-bisphosphate is cleaved by aldolase A yielding glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, meanwhile dehydrogenase glyceroaldehydephosophatase catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (Scheffler & Gerrard, 2007). Both enzymes are part of the glycolytic system and structural changes of these proteins would probably affect post-mortem metabolism. Furthermore, denaturation of both enzymes have previously been related to variation in the metabolic rate post-mortem (Molette, Remignon, & Babile, 2005).

Table 1
Identification of proteins bound to bisANS by MALDI-TOF MS

Band number	Identified fragments by MALDI-TOF	MW (kDa)	Database NCBInr	Calculated MW (Da)
3	Muscle glycogen phosphorilase (*sus scrofa*)	94	gi/106073338	84382
4	Muscle 6-phosphofructokinase (*sus scrofa*)	82	gi/95117652	82443
6	Pyruvate kinase, isozyme MI	58	gi/75061500	58494
7[a]	Chain A, crystal structure of pig phosphoglucose isomerase	58	gi/21730305	63126
	Chain pyruvate kinase Mol_id. M1 pyruvate kinase		gi/1311281	58391
9	Creatin kinase M	44	gi/180576	43247
10	Similar to fructose-bisphosphate aldolase A (muscle-type aldolase)	41	gi/76653611	39925
11	Glyceraldehyde-3-phosphate dehydrogenase	36	gi/229279	35915
12, 13	L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) LDH-M	34	gi/1171740	36880
14	Carbonic anhydrase III (*sus scrofa*)	29	gi/56711366	29678

[a] Corresponding to a protein mixture.

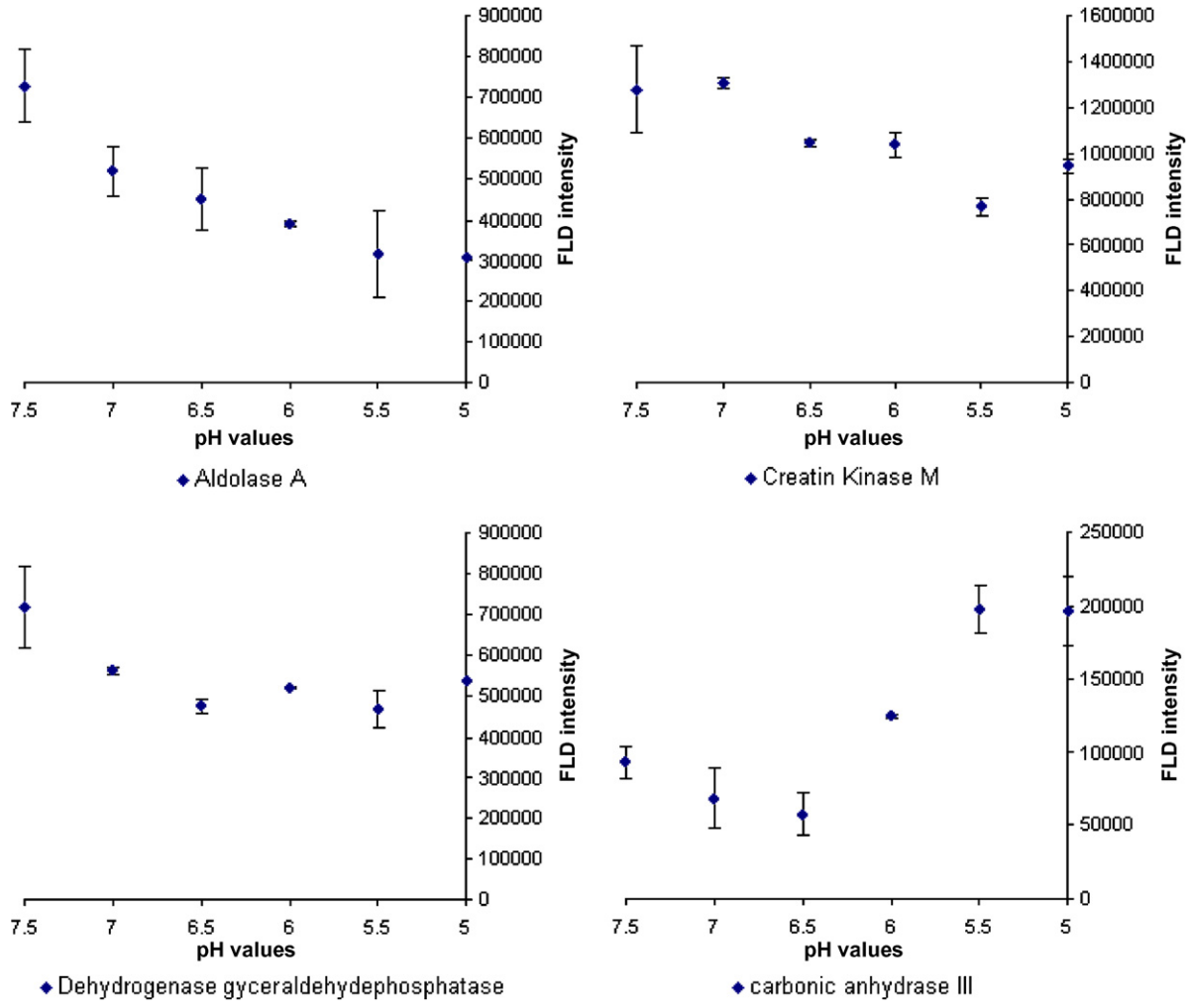

Fig. 3. BisANS–protein fluorescence in the pH range (7.5–5.0) and at 25 °C of the indicated sarcoplasmic proteins.

Creatine kinase catalyzes the conversion of creatine to phosphocreatine, consuming adenosine triphosphate (ATP) and generating adenosine diphosphate (ADP) and the reverse reaction. In tissues that consume ATP rapidly, phosphocreatine serves as an energy reservoir for the rapid regeneration of ATP, the major source of energy in biochemical reactions (Cook, Kenyon, & Cleland, 1981). It was also found that creatine kinase was more denaturated in fast glycolysing pigs compared with normal glycolysing pigs (Ryu et al., 2005).

Finally, carbonic anhydrase III is a metalloenzyme that catalyzes the rapid interconversion of carbon dioxide and water to carbonic acid and protons and it also converts bicarbonate in carbon dioxide and water, being important in regulation of the cellular pH (Duda et al., 2005). In contrast to the other proteins that were found to decrease in

the fluorescence intensity with decreasing pH, carbonic anhydrase III had an increase in fluorescence intensity with decreasing pH, maybe as a consequence of the formation of a stable complex with increased surface hydrophobicity. The sarcoplasmic proteins muscle glycogen phosphorilase, muscle 6-phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase did not seem to be affected by the pH changes with this screening method, their bands presenting similar fluorescence at the different pH's. However conformational changes due to pH cannot be discounted.

No apparent differences due to temperature, 35 °C versus 25 °C were observed (Figs. 1 and 2). When the sarcoplasmic proteins were incubated at 40 °C or higher, cross-linking of proteins was observed. These results were probably caused by a high degree of protein denaturation (data not shown). These results indicate that there is a narrow threshold, around 40 °C where marked protein denaturation is caused by an increase in temperature. This observation is in agreement with studies made on myofibrillar proteins that showed no changes in protein hydrophobicity at 30 °C. However, at 40 °C and above there was a significant difference after 15 min of incubation (Chelh, Gatellier, and Sante-Lhoutellier, 2006).

4. Conclusions

The results of the present study suggest that the use of the hydrophobic probe bisANS is a suitable method to screen for changes in the hydrophobic domain of proteins in a complex mixture.

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