Food Chemistry 138 (2013) 679–686

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterising protein, salt and water interactions with combined vibrational spectroscopic techniques

Nebojsa Perisic [a,b,*], Nils Kristian Afseth [a], Ragni Ofstad [a], Sahar Hassani [a,b], Achim Kohler [a,c]

[a] Nofima AS, Osloveien 1, N-1430 Aas, Norway
[b] Institute of Mathematical Sciences and Technology, Norwegian University of Life Sciences, PO Box 5003, 1432 Aas, Norway
[c] Centre for Integrative Genetics (CIGENE), Department of Mathematical Sciences and Technology (IMT), Norwegian University of Life Sciences, 1432 Aas, Norway

ARTICLE INFO

Article history:
Received 5 August 2012
Received in revised form 11 October 2012
Accepted 16 October 2012
Available online 12 November 2012

Keywords:
FTIR
Raman
NIR
CPCA
Multi-block
Protein secondary structure
NaCl
KCl
$MgSO_4$
Amino acid residues

ABSTRACT

In this paper a combination of NIR spectroscopy and FTIR and Raman microspectroscopy was used to elucidate the effects of different salts (NaCl, KCl and $MgSO_4$) on structural proteins and their hydration in muscle tissue. Multivariate multi-block technique Consensus Principal Component Analysis enabled integration of different vibrational spectroscopic techniques: macroscopic information obtained by NIR spectroscopy is directly related to microscopic information obtained by FTIR and Raman microspectroscopy. Changes in protein secondary structure observed at different concentrations of salts were linked to changes in protein hydration affinity. The evidence for this was given by connecting the underlying FTIR bands of the amide I region (1700–1600 cm^{-1}) and the water region (3500–3000 cm^{-1}) with water vibrations obtained by NIR spectroscopy. In addition, Raman microspectroscopy demonstrated that different cations affected structures of aromatic amino acid residues differently, which indicates that cation–π interactions play an important role in determination of the final structure of protein molecules.

1. Introduction

Interactions between the conformational states of proteins, water molecules and salt ions have been a focus of numerous studies (Barrett, Peticolas, & Robson, 1978). As proteins and their conformational states possess a variety of biological activities, these interactions have important biological implications. For muscle-based foods, salting is known to affect a variety of parameters, such as tenderness and processability, which largely could be ascribed to complex interactions of proteins, water and salts (Ruusunen & Puolanne, 2005). There are continuing efforts to reduce the content of sodium in foods and inevitably NaCl due to its adverse effects on human health (He & MacGregor, 2008). Understanding the interaction processes of various salts and food components is thus of great importance.

While protein molecules exhibit an unrestricted complexity in terms of molecular structure, the ions of inorganic salts and water molecules could be regarded as rather simple entities. Although the influence of ions on protein stability was discovered and classified empirically by Hofmeister (1888), their interactions are far from being fully elucidated and characterised. This is largely due to the diverse and versatile properties of water molecules (Zhang & Cremer, 2010). Such interactions are more easily studied in pure protein model systems while biological systems present challenges to the complete understanding of interactions within complex protein matrices, even in refined systems such as gelatin gels (Yang, Wang, Regenstein, & Rouse, 2007).

The charges of the ions are bound to affect the way ions interact with protein surfaces. It has been shown that anions accumulate on the charged surface of the protein and consecutively influence the protein conformational structure (Kamerzell, Esfandiary, Joshi, Middaugh, & Volkin, 2011). The interaction of cations with protein molecules, on the other hand, is often explained by cation–π interactions (Dougherty, 2007) and is postulated to be mainly localised on the amino acid residues. Cation–π interaction can be visualised as an electrostatic interaction between the cation and the quadrupole moment of an aromatic molecule, which is contained in several amino acids. The explanation for this non-covalent molecular interaction is partially based on *ab initio* molecular

* Corresponding author at: Nofima AS, Centre for Biospectroscopy and Data Modelling, Osloveien 1, N-1430 Aas, Norway. Tel.: +47 64 97 01 00; fax: +47 64 97 03 33.
E-mail address: nebojsa.perisic@nofima.no (N. Perisic).

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http://dx.doi.org/10.1016/j.foodchem.2012.10.117

orbital calculations and it states that the negative charge is localised in the centre of the aromatic ring, with a belt of positive charge around the ring edges (Dougherty, 2007). Cation–π interaction seems important, particularly when it involves aromatic amino acid residues of tryptophan, tyrosine and phenylalanine. These are essential and among the most abundant amino acids in mammalian muscles (Beach, Munks, & Robinson, 1943).

In order better to portray the picture of the protein–water–salt interactions it is highly desirable to be able to follow, simultaneously and *in situ*, as many of the important interactions in one biological system. Vibrational spectroscopy constitutes a set of powerful tools that have been frequently used for studying these interactions (Boecker, Ofstad, Bertram, Egelandsdal, & Kohler, 2006; Kamerzell et al., 2011). A particularly powerful feature of FTIR and Raman spectroscopy is the analysis of protein secondary structure (Bertram, Kohler, Boecker, Ofstad, & Andersen, 2006; Jackson & Mantsch, 1995). Recent studies have shown that NIR spectroscopy can likewise be used to follow protein unfolding and protein–lyoprotectant hydrogen bond interactions by monitoring changes in the amide A/II band (around 2062 nm) (Pieters et al., 2012). Furthermore, Raman spectra are known to be rich sources of information on amino acid residues (Overman & Thomas, 1999), and several different processes, such as the water exposure of buried tyrosine, could be monitored by studying changes in the Raman amino acid vibrations (Herrero, 2008). Vibrational spectroscopy has also been successfully utilised in applications such as the investigation of hydration processes of bovine serum albumin and lysozyme (Liltorp & Maréchal, 2005), interactions of proteins with various types of ligands and excipients (Barth, 2007; Kamerzell et al., 2011), specific influences of various inorganic salt on conformational changes in myosin (Barrett et al., 1978) and probing of enzymatic activities (Barth, 2007).

In most cases of FTIR analyses, water needs to be removed from samples, frequently by cryo-sectioning and snap drying, due to its strong absorption in the protein region (Boecker et al., 2007; Perisic, Afseth, Ofstad, & Kohler, 2011). NIR spectroscopy, on the other hand, provides a means for the direct analysis of the native matrix. The technique provides information on combinations of the fundamental vibrations as well as their overtones (Williams & Norris, 1987). Correspondingly, NIR spectroscopy has been successfully applied in various studies for the quantification and prediction of main food constituents as well as for predicting certain qualities, such as water binding capacity (WHC) and pH value (Ripoll, Albertí, Panea, Olleta, & Sañudo, 2008). Qualitative interpretations of NIR spectra still represent an open and challenging field (Williams & Norris, 1987).

Due to dissimilarities between the information obtained by NIR on one side and FTIR and Raman on the other, direct links between the spectra of the respective techniques are seldom reported. However, by integrating multiple layers of information, a more complete insight into the properties of complex biological systems can be achieved. The aim of the present study is to combine spectra of three vibrational spectroscopic techniques, namely FTIR, Raman and NIR spectroscopy, to obtain an overall picture of salt-protein-water interactions on a molecular level. Consensus principal component analysis (CPCA) was used to combine the three spectroscopic techniques. CPCA is capable of integrating multivariate signals from different measurement techniques in one data model and allows investigation of the relations between the molecular signatures traced by different techniques. The investigated biological system consisted of bovine meat tissue samples exposed to NaCl, $MgSO_4$, and KCl and their mixtures at different concentrations (Perisic et al., 2011).

2. Materials and methods

2.1. Brining

Samples of beef muscle (*longissimus dorsi)* were taken from four Norwegian Red cattle 48 h post rigour. The samples were obtained from a commercial slaughter-house. From each animal, two muscle blocks of approximately $4 \times 4 \times 1$ cm were excised and placed in each of nine different salt brines. The salt brines were comprised of pure NaCl, KCl and $MgSO_4$ solutions, made in 1.5%, 6% and 9% total salt weight percentage concentration, respectively. To avoid dilution of the salt brines, the mass ratio of meat to brine was set to approximately 1:8. The samples were kept in brines at 4 °C for 48 h with 0.05% NaN_3 added in order to prevent any possible deterioration caused by microbial growth (Perisic et al., 2011).

2.2. NIR spectroscopy

Immediately after brining, samples were analysed by NIR spectroscopy without further preparation. VIS/NIR spectra were obtained by a NIRSystems XDS Rapid Content Analyzer (Foss NIRSystems, Silver Spring, MD, USA) equipped with a quartz halogen lamp and a PbS/Si detector. The spectra were collected in the reflectance mode employing an internal ceramic reference standard. The spot size of the incoming light was set at 9.5 mm (diameter), and all spectra were acquired in standard sample cups with quartz windows. Each spectrum was the average of 32 scans, and all spectra covered the spectral region of 400–2500 nm (with a digital resolution of 0.5 nm). The final NIR data set consisted of four replicate spectra per animal and brine, resulting in 144 spectra (nine brines × four animals × four replicates).

2.3. Microspectroscopy

For FTIR and Raman microspectroscopic measurements, two muscle blocks of approximately $1.0 \times 0.6 \times 0.3$ cm were excised from each of the muscle samples, consecutively embedded in O.C.T. compound (Tissue-Trek, Electron Microscopy Sciences, Hatfiles, USA), and snap-frozen in liquid N_2. Afterwards, all samples were transferred to a −80 °C freezer where they were stored until cryo-sectioning, which was performed transversely to the fibre direction on a Leica CM 3050 S cryostat (Leica Microsystems Wetzlar GmbH, Wetzlar Germany). From each of the snap-frozen meat pieces four cryo-sections were excised, two for FTIR analysis and two for Raman analysis.

2.3.1. FTIR microspectroscopy

Detailed FTIR analysis and sample preparation are provided in our previous study (Perisic et al., 2011). The resulting FTIR data set consisted of 30 single-myofibre spectra for each animal and experimental treatment. The final data set consisted of 1080 spectra (30 replicate spectra × four animals × nine brines).

2.3.2. Raman microspectroscopy

For Raman microspectroscopy, the cryo-sections were cut in 20 μm thickness, thaw-mounted on CaF_2 slides and subsequently stored in a desiccator before acquisition of the Raman spectra. Raman spectra were recorded by a LabRam HR 800 Raman microscope (Horiba Scientific, France). The excitation wavelength of 632.8 nm was generated by a He–Ne laser. A 100× objective (Olympus, France) was used for focusing and collecting scattered Raman light. The laser power was approximately 15 mW on the sample surface. The confocal hole was set at 200 μm and an exposure time of 4×15 s was used. The Raman scattering was dispersed with a 300 lines/mm grating, which resulted in spectra in