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A novel fluorimetric assay for visualization and quantification of protein carbonyls in muscle foods

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ABSTRACT

Muscle foods, particularly fish products are highly exposed to oxidative stress during processing and storage, resulting in oxidative modification of proteins. Protein carbonyls content has been used as one of the measures of oxidative stress. Generally, the resulting carbonylated proteins (CPs) have so far been labeled with 2,4-dinitrophenyl (DNP) hydrazine and detected with anti-DNP antibody. However, the applicability of this method to food samples is limited by its high price, time-consuming procedure and possibility to perform the measurements just on soluble protein fractions. We developed a simpler, faster and cheaper method to assess CP level in muscle foods, including both soluble and insoluble protein fractions, which is based on a direct reaction of protein carbonyls with 7-(diethylamino)coumarin-3-carbohydrazide (CHH). The paper describes a novel technique to label both soluble and insoluble carbonylated proteins with CHH and determine carbonyl content by fluorescence microscopy assay which correlates (R = 0.911) with conventional ELISA method.

1. Introduction

Oxidative degradation of proteins in muscle foods such as meat and fish is currently one of the major directions of research due to its undesired impacts on the product quality. Protein oxidation reactions are known to cause decrease in sensory and nutritional quality, as well as unwanted changes in textural characteristics of muscle foods (Lund & Baron, 2010; Standal et al., 2018; Cropotova, Mozuraityte, Standal, Aftret, & Rustad, 2019). New preservation methods aiming to fulfil the growing preferences of consumers towards fresh and high-quality foods require deeper comprehension of chemical and biochemical reactions during application of novel processing technologies. Thus, studying the phenomenon of protein oxidation and methods for its reliable detection continues to be central focus for food researchers and professionals.

In recent years, an increased attention has been paid to new methods for detection of non-enzymatic, free radical-mediated oxidation of proteins (Rogowska-Wrzesinska, Nedić, Baron, & Griffiths, 2014; Augustyniak et al., 2015). The effects of protein oxidation in muscle foods are deleterious, leading to both structural and functional alterations of protein molecules. The chemical path for protein oxidation and the resulting biological consequences depend upon several factors, as follows: 1) the primary structure; 2) whether or not the oxidant can have access to susceptible amino acid residues within a protein molecule i.e. three dimensional structure constraints and 3) the oxidising species (Nystrom, 2005; Augustyniak et al., 2015). Another carbonylation pathway is a metal-catalyzed free radical attack on the amino acid side chains of lysine, arginine, proline and threonine (Dalle-Donne et al., 2006). Moreover, carbonyl derivatives can also be formed through the α-amidation pathway (Dalle-Donne et al., 2006; Rogowska-Wrzesinska et al., 2014).

Carbonylated protein molecules can be analyzed by a number of analytical techniques based on a specific chemical derivatization of carbonyl groups with hydrazines, hydrazides and hydroxylamines (Yan & Forster, 2011; Vemula, Ni, & Fedorova, 2015) which can be detected spectrophotometrically (Levine et al., 1990), by using ELISA kit (Uehara & Rao, 2015) or Western blot method (Luo & Wehr, 2009). Other analytical methods such as chromatography (Levine, Williams, Stadtman, & Shacter, 1994; Bollineni, Hoffmann, & Fedorova, 2011), mass spectrometry (Baraibar, Ladouce, & Friguet, 2013) and fluorescence spectroscopy (Tamarit et al., 2012; Baraibar et al., 2013) can also be applied to perform quantification of carbonylated molecules. For the spectrophotometric method of quantification of carbonylated proteins, 2,4-dinitrophenylhydrazine (DNPH) is generally used as a derivatization reagent (Levine et al., 1990). Presence of anti-DNP anti-

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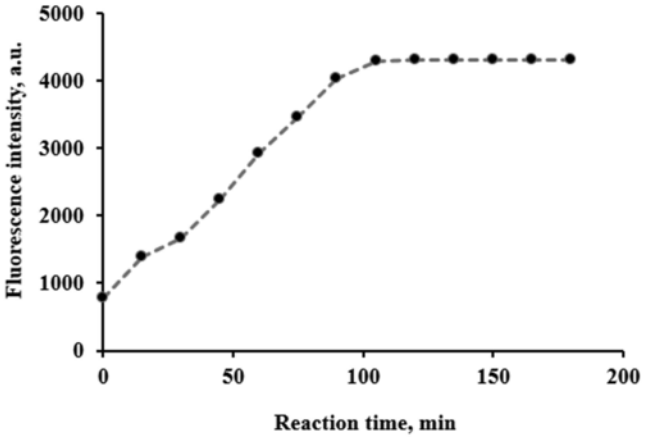

Fig. 1. Kinetic reaction of CHH with BSA-standards (example is shown for carbonyl content 6 nmol/mg protein).

Table 1
Total carbonyl content measured in BSA-standards by conventional ELISA method and developed fluorimetric assay based on CHH-labelling of carbonyls.

Sample type	Protein carbonyls by ELISA (nmol/mg)	Protein carbonyls by novel fluorometric assay with CHH without washing with PBS (nmol/mg)	Protein carbonyls by novel fluorometric assay with CHH after washing with PBS (nmol/mg)
BSA-standards	1.50 ± 0.01	1.90 ± 0.02	1.50 ± 0.04
	3.00 ± 0.01	3.63 ± 0.11	3.02 ± 0.05
	4.50 ± 0.04	5.04 ± 0.11	4.58 ± 0.08
	6.00 ± 0.01	7.24 ± 0.02	6.93 ± 0.03
	7.50 ± 0.01	8.34 ± 0.12	7.25 ± 0.05

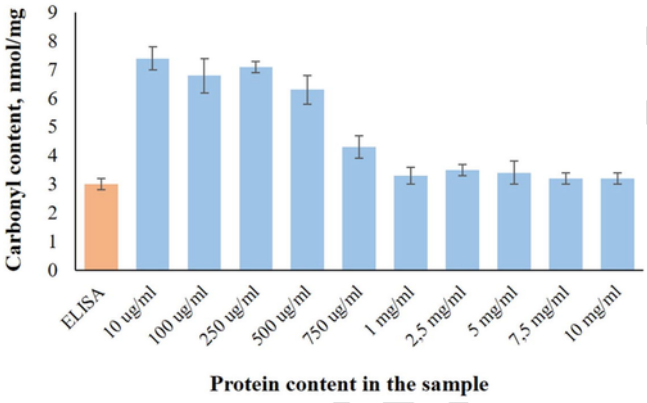

Fig. 2. Total carbonyl content measured in BSA-standards with protein content ranging from 10 μg/ml to 10 mg/ml by conventional ELISA method and developed fluorimetric assay based on CHH-labelling of carbonyls.

bodies allows converting it into a sensitive immunodetection assay by ELISA or Western blots (Uehara & Rao, 2015; Vemula et al., 2015). However, standard DNPH-based immunochemistry protocols including ELISA and Western blots are laborious, time-consuming, expensive, may suffer from cross-reactivity of antibodies and do not allow evaluation of spatial distribution of carbonylated proteins.

Despite rapid advances in high-throughput protein analyses, determination of protein carbonyls in insoluble protein fractions such as membrane and scaffold proteins, has been a major challenge in biological and food science, and so far remains a bottleneck in quantitative analysis (Geumann, Grønborg, Hellwig, Martens, & Jahn, 2010). Accumulation of carbonyls is a dynamic process occurring under oxidative stress and thus spatial cellular and tissue carbonyl distribution in both soluble and insoluble protein fractions might change over time. However, cellular and tissue localization of carbonylated species has only been studied in soluble protein fractions by fluorometric assays involving fluorescent labelling of proteins by cyanine, BODIPY, and coumarin-hydrazide (Vemula et al., 2015). Therefore, it is important to develop new methods to analyse carbonyl spatial distribution and content in muscle foods to attribute carbonyl levels to certain soluble and insoluble protein species, such as it was done by two-dimensional gel electrophoresis detection technique coupled with DNPH-labelling for soluble protein fractions (Conrad et al., 2001; Kjærsgård & Jessen, 2004; Yoo & Regnier, 2004).

In this paper, the focus is put on determination of protein carbonyls in both soluble and insoluble protein fractions of fish samples (insoluble protein scaffolds, etc.). The method development was motivated by our interest in total protein carbonylation assessment in muscle foods, particularly fish products. For the assay development, a direct fluorescent labelling of protein extracts by coumarin-hydrazide was used. The proposed fluorescence microscopy assay represents a rapid, non-invasive and cost-efficient method aiming to not only quantify carbonyls in both soluble and insoluble proteins, but also provide information on their spatial localization and distribution in the tissue by fluorescence imaging. The protocol was verified for proteins extracted from different fish species and compared with DNPH-based immunocytochemistry. Thus, a novel fluorescence non-invasive detection method proposed in the study can help estimate the carbonylation extent in both soluble and insoluble proteins, considerably reducing sample amounts in comparison with existing methods such as ELISA or Western blotting.

2. Materials and methods

2.1. Reagents and materials

The commercial ELISA kit, STA-310 OxiSelectTM used for determination of protein carbonyls in biological samples was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). All other chemicals and reagents used for protein carbonyl detection and quantification were of the highest grade available, and were purchased from Sigma–Aldrich (Germany).

Minced fillets of haddock (*Melanogrammus aeglefinus*), Atlantic mackerel (*Scomber scombrus*) cod (*Gadus morhua*) and salmon (*Salmo salar*) were used as raw material in the study for validation of the developed fluorimetric assay. The fish fillets were purchased from a local retailer (Ravnkloa, Trondheim, Norway), minced using a benchtop blender (Bosch, Germany) and placed in polyethylene bags (portions of 500 g fish mince per bag, oxygen permeability 25 cm^3/m^2) for frozen storage at −20 °C for 1 month.

Before protein carbonyl analyses, the fish fillets were defrosted in a cold room at 4 ± 1 °C overnight. Analyses were performed in three replicates before and after freezing and the average value with standard deviation were calculated.

2.2. Chemical and physical assays

2.2.1. ELISA assay with commercial kit

Protein carbonyl groups were determined by DNPH-based Enzyme-Linked Immunosorbent Assay (ELISA) in a 96-well polystyrene plate as a measure of protein oxidation (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997).

Briefly, extracts of water- and salt-soluble proteins obtained from fish minces by a modification of the method of Licciardello et al. (1982), as previously described by Hultmann and Rustad (2002), were used to determine total carbonyls in sarcoplasmic and myofibrillar pro-

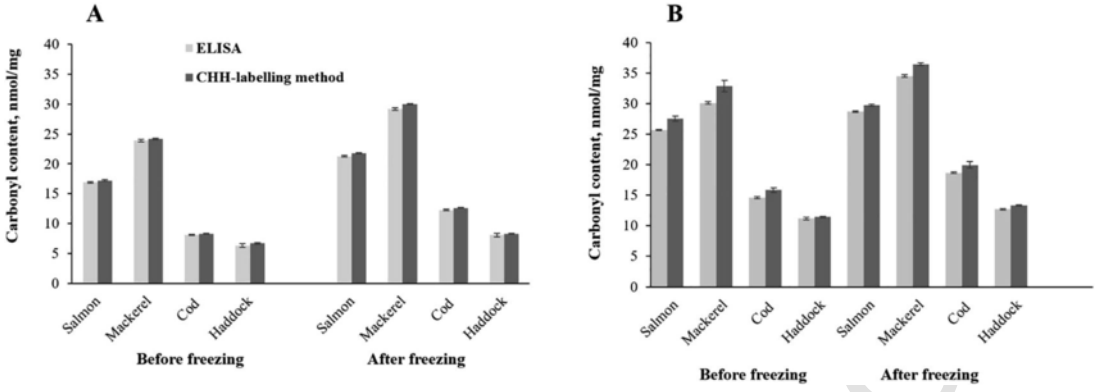

Fig. 3. Total carbonyl content measured in fish samples by conventional ELISA method and developed fluorimetric assay based on CHH-labelling of carbonyls.

teins. Before the analysis, all samples and oxidized and reduced bovine serum albumin (BSA) standards were diluted with 1X PBS to obtain solutions with protein concentration 10 μg/ml, and then 100 μl of each sample were introduced in a 96-well protein binding plate for incubation overnight at 4 °C. Then, each well was washed three times with 250 μl 1X PBS, and 100 μl DNPH working solution were added followed by incubation for 45 min at room temperature under dark.

After this, the wells were washed five times with 250 μl 1X PBS/ethanol (1:1, v/v) with a 5-minute incubation on an orbital shaker with 5 min between washes. Further, 200 μl ELISA blocking solution were added in each well followed by a 2-hour incubation at room temperature on an orbital shaker. After the incubation, the wells were washed three times with 250 μl ELISA wash buffer and 100 μl diluted anti-DNP antibody were added followed by one-hour incubation at room temperature on an orbital shaker and subsequent washing with ELISA wash buffer. Further, 100 μl diluted HRP-conjugated secondary antibody were added to the wells, and the same washing procedure was performed for five times. Then, 100 μl ELISA substrate solution were added in each well and incubated at room temperature on an orbital shaker for approximately 15 min. The enzyme reaction was ended by adding 100 μl ELISA stopping solution in all wells, and the absorbance was read immediately on a multimode microplate reader (Spark, Tecan, Austria) at 450 nm.

Carbonyl groups were determined in the three parallels for each protein extract and the average value with standard deviation were calculated. Carbonyl content was calculated from the standard curve and the results were expressed as nanomol carbonyl per milligram of protein.

2.2.2. Fluorometric method based on 7-(diethylamino)-coumarin-3-carbohydrazide (CHH) labeling

The proposed fluorescence assay is based on 7-(diethylamino)-coumarin-3-carbohydrazide (CHH) properties to fluorescencently label carbonyl groups of proteins (Vemula et al., 2015). CHH is a versatile fluorescent chemical dye (λ_{ex} = 400–420/λ_{em} = 468 nm) possessing good solubility, efficient cell permeability and high fluorescent efficiency with a characteristic blue light emission in the wavelength region of DAPI dyes (Vemula et al., 2015).

2.2.2.1. Determination of total carbonyls in extracts of water- and salt-soluble proteins Briefly, extracts of water- and salt-soluble proteins of fish minces were properly diluted with 10 mM phosphate-buffered saline (1X PBS, pH 7.4) to adjust concentration of protein to 1–5 mg/ml. Commercially prepared reduced and oxidized BSA standards provided by the OxiSelect Protein Carbonyl ELISA kit (STA-310, Cell Biolaboratories) were used for the standard curve preparation.

Briefly, 200 μl of the prepared protein samples (1 mg/ml) and oxidised and reduced BSA standards were added to a 96-well protein binding plate and incubated at 37 °C for 3 h to adsorb to the plate's wells due to hydrophobic interaction between them and the plastic well plate. Then, the wells containing samples and the standards were washed 3 times with 250 μl 1X PBS per well. After the last wash, the wells were tapped on a paper towel to remove excess of PBS, and stained with 7-(diethylamino)-coumarin-3-carbohydrazide (CHH). For CHH-labelling, samples were previously blocked in ELISA blocking solution (5% FBS, 0.1%Tween-20 in PBS) for 1 h at room temperature on an orbital shaker. The wells were then washed 3 times with 250 μl washing buffer (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween-20) to remove excess of blocking reagent followed by tapping wells on a paper towel after the last wash. Further, 100 μl of 0.2 mmol/L CHH in methanol was added to each well following incubation for 2 h at room temperature on a plate shaker. The wells were then washed 3 times with 1X PBS and fluorescence was recorded at λ_{ex} = 420/10 and λ_{em} = 460/20 nm by using Spark multimode microplate reader (Tecan, Austria).

For fluorescence imaging, the samples and BSA-standards were placed with a Pasteur pipette on glass microscope slides after incubation with CHH, covered by cover slips and directly visualized on a Zeiss Axio Imager Upright microscope equipped with Zeiss Plan-Apochromat 10× objective and AxioCam ERc5s with a filter set 49 DAPI (EX 365/50, FT 395, EM 445/70). The acquired micrographs were processed using a classical image-processing protocol of the ImageJ software, and the total fluorescence intensity was measured, as described in Cropotova, Mozuraityte, Standal, and Rustad (2018).

Carbonyl content was calculated from the standard curve prepared from oxidised and reduced BSA-standards, and expressed as nanomol carbonyl per milligram of protein.

2.2.2.2. Determination of total carbonyls in insoluble protein fractions Proteins that are not soluble in water or salt are mainly connective tissue proteins (i.e. stroma proteins, collagen, elastin). Direct fluorescence visualization of carbonyls in insoluble protein fractions of the fish minces was performed on a Zeiss Axio Imager Upright microscope. Briefly, 10 g of fish mince were soaked in 0.1 M NaOH with a sample/solution ratio of 1:10 (w/v) for 24 h, with a change of solution every 6 h to remove non-connective tissue proteins. The resulting sample was then washed with distilled water until the drained water reached a neutral pH and centrifuged at 1500*g* for 20 min using a Heraeus Multifuge X1 (Thermo Scientifc, USA) to separate the remaining lipid fraction. Isolated connective tissue proteins collected after the treatment were washed with 1X PBS, placed on glass microscope slides, blocked in ELISA blocking solution (5% FBS, 0.1%Tween-20 in PBS) for 1 h at room temperature and further subjected to CHH-labeling (0.2 mmol/L) for 2 h at room temperature in the darkness. After incubation with CHH, the insoluble fish tissue fibrils were washed with warm PBS, covered by cover slips and imaged immediately under fluorescence mode by using a Zeiss Plan-Apochromat 20× objective with a filter set 49 DAPI (EX 365/50, FT 395, EM 445/70). The mean fluorescence in-

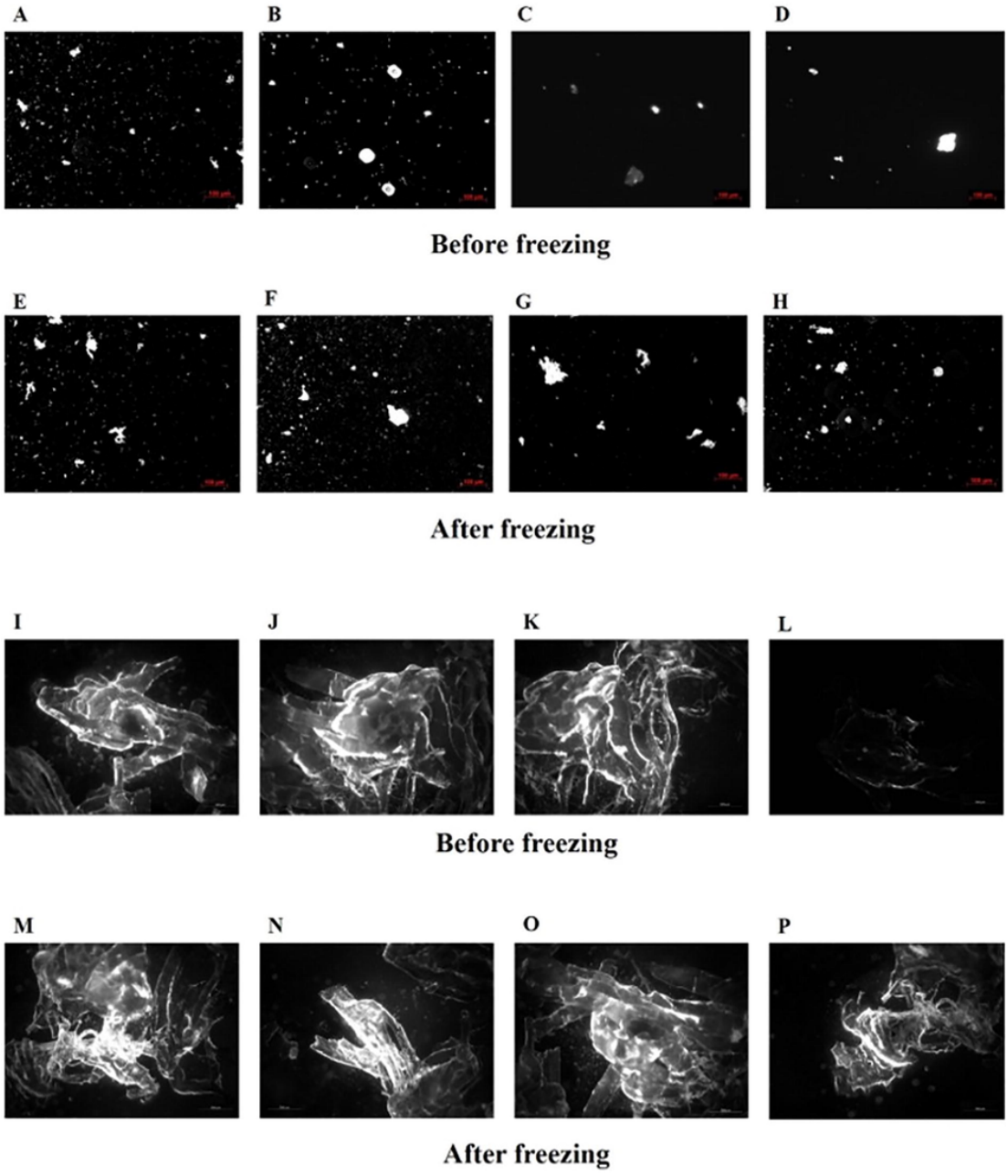

Fig. 4. Fluorescence visualization of CHH-labelled carbonyls in sarcoplasmic protein extracts of fish samples before freezing: A – salmon; B – mackerel; C – cod; D – haddock; after freezing: E – salmon; F – mackerel; G – cod; H – haddock; and in connective tissue proteins before freezing: I – salmon; J – mackerel; K – cod; L – haddock; and after freezing: M – salmon; N – mackerel; O – cod; P – haddock.

tensity (MFI) of connective tissue proteins in the acquired images was determined after subtraction of background. The image processing system used was a software package ImageJ, v1.51k 1 (National Institutes of Health, Bethesda, MD, USA). The acquired micrographs were processed using a classical image-processing protocol of the ImageJ software, comprising background subtraction, automatic threshold and binary filter, and the resulting fluorescent areas were analyzed on the mask image (Cropotova et al., 2018). Carbonyl content was calculated from the standard curve constructed by mixing varying proportions of reduced and oxidized bovine serum albumin (BSA) as described above, and the concentration of protein carbonyls in insoluble protein fractions was expressed as nanomoles of carbonyl moieties per milligram of protein (nmol/mg).

2.3. Statistical analysis

All experiments were conducted in triplicate, and data were reported as the mean ± standard deviation. Simple correlations among the studied parameters were evaluated by the Pearson coefficient by using SigmaPlot software, version 14 (Systat 183 Software Inc. USA). When appropriate, the developed fluorometric assay was compared with the conventional ELISA method using a paired Student's *t* test. The criterion for significance was set at $P < 0.05$.

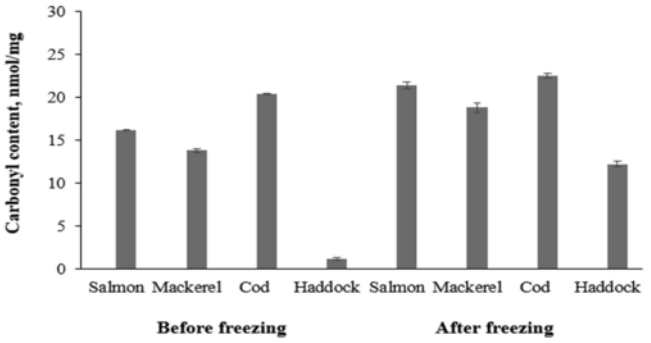

Fig. 5. Total carbonyls content in connective tissue proteins of fish minces obtained by the developed fluorimetric assay based on CHH-labelling of carbonyls.

3. Results and discussion

3.1. Development of a fluorometric assay for protein carbonyls measurement in a microplate format

Direct measurement of protein carbonyls in biological samples cannot be performed due to lack of their intrinsic UV/visible spectrophotometric absorbance/fluorescence properties (Stocker et al., 2015). Therefore, the conventional methods involve derivatization of carbonyl moieties in proteins with DNPH and their subsequent detection with anti-DNP antibodies (Buss et al., 1997; Alamdari et al., 2005). However, classical immunodetection assays such as ELISA or Western blots used for detection and quantification of protein carbonyls are expensive, laborious and do not allow measuring carbonyls in insoluble protein fractions. Moreover, some proteins such as cytochrome *c* and hemoglobin have absorbance wavelengths similar to DNPH, which can interfere with carbonyl measurement (Stocker et al., 2015).

Therefore, an alternative fluorometric assay for rapid visualization and quantification of protein carbonyls was proposed in the study. 7-(Diethylamino)coumarin-3-carbohydrazide (CHH) was selected as a good alternative of carbonyl label for the direct measurement of protein carbonyls due to its high fluorescence signal and sensitivity compared to DNPH (Vemula et al., 2015). Thus, we suggest direct detection and quantification of protein carbonyls in muscle foods and other biological samples by converting the fluorescence signal arising from CHH-labeled proteins in analyzed samples to nanomoles by using a CHH-standard curve.

The validity of the proposed fluorometric assay depends on several factors, including: purity of protein fraction (without remaining lipid fractions containing lipid carbonyls), possibility of the fluorescence dye (CHH) to quantitatively react with all protein carbonyls, removal of unreacted CHH by special washing steps, and existence of valid standards for CHH calibration curve.

To compare the sensitivity of the proposed fluorimetric carbonyl assay and ELISA method, various dilutions of CHH dye (concentration range 0.1–0.8 mmol/L) for labelling of reduced and oxidized BSA standards were analyzed (data not shown). It was revealed that the optimal concentration of CHH lies in the range of 0.2–0.4 mmol/L (precision 4.68% compared to ELISA). Therefore, it was further decided to use CHH concentration of 0.2 mmol/L in the method development, as stated in *Materials and Methods*.

To identify the appropriate time required for CHH to react with all protein carbonyls present in the sample, while removing unreacted CHH, we tested different reaction times. The kinetics of the reaction of CHH with oxidized and reduced BSA standards was examined as described in *Materials and Methods*. As shown in Fig. 1, the CHH-labelling reaction was nearly complete after 2 h. Thus, after 120 min of incubation with CHH, a plateau was reached and the fluorescence intensity was stable for more than 2 h (Fig. 1). Therefore, it was further decided to perform fluorescence scanning at a plate reader or fluorescence microscope after 2-hour staining.

However, because the plate reader detected additional fluorescence coming from unreacted CHH (Table 1), we had to include a procedure for complete removal of free CHH from the CHH-linked proteins. As shown in Table 1, this was fully accomplished by PBS washing step described in *Materials and Methods*.

3.2. Repeatability and validation of the developed fluorometric assay in a microplate format

To check the repeatability of results for the proposed fluorimetric assay, protein carbonyls were measured in a series of samples with total protein concentration ranging from 10 μg/ml to 10 mg/ml, containing various amounts of oxidized and reduced BSA, by both the CHH-labeling assay and conventional ELISA method. The repeatability measurements comprised all steps from the sample preparation to the final measurement of the outcome, and the protein carbonyl content was expressed as nmol/mg protein. The mean values of protein carbonyls for each sample obtained by these two assays are displayed in Fig. 2. It can be clearly seen, that there is a significant correlation ($p < 0.05$, $R = 0.910$) between the fluorimetric CHH-assay and ELISA method for the measurement of protein carbonyls in the samples with protein concentrations 1–10 mg/ml. However, the coefficient of variation is significantly ($p < 0.05$) increasing with a decrease in protein content of the sample below 1 mg/ml.

To test the validity of the developed fluorometric assay with biological samples, we measured protein carbonyls in protein extracts obtained from different fishes by both CHH-labeling and ELISA methods. As shown in Fig. 3, the mean carbonyl content in the analyzed fishes determined by the developed fluorometric assay is slightly higher than the mean value obtained by the conventional ELISA method, but the difference is not statistically significant. According to our hypothesis, this phenomenon can be explained by some remaining fluorescent signal coming from free CHH after the washing steps.

3.3. Repeatability and validation of the developed fluorometric assay for fluorescence microscopy format

To check the repeatability of results and validate the developed fluorometric assay for fluorescence microscopy format, CHH-labelled carbonyls in BSA-standards and protein extracts of fish minces were transferred from a 96-well microplate on glass microscope slides, covered by cover slips and directly visualized on a Zeiss Axio Imager Upright microscope as described in *Materials and Methods*. The carbonyl content was calculated from the standard curve constructed on the basis of mean fluorescence intensity coming from BSA-standards and the obtained results were compared with the developed fluorometric microplate assay and conventional ELISA method. The mean values of protein carbonyls for each sample obtained by these assays are displayed in Fig. 3, and the visualization of protein carbonyls is shown in Fig. 4A-H. It is clearly seen that both salmon and mackerel samples had significantly ($p < 0.05$) higher carbonyl content values in comparison with cod and haddock minces after 1 month of frozen storage in both the sarcoplasmic and myofibrillar proteins (Figs. 3 and 4A-H). This phenomenon can be explained by higher lipid content in salmon and mackerel compared to cod and haddock. Thus, freezing resulted in the rupture of cell membranes of adipocytes, leading to liberation of lipid oxidation products which further interacted with proteins, generating protein carbonyls (Standal et al., 2018). Another possible explanation of increased protein carbonylation is due to release of free iron from dark muscle of the fishes during mincing and subsequent frozen

storage. Considering the fact, that dark muscle of Atlantic mackerel and salmon is rich in haem proteins, its mechanical and low-temperature degradation may result in increased protein carbonylation due to a release of free iron (Tokur & Korkmaz, 2007). Thus, increased protein carbonylation in the analyzed mackerel and salmon minces is probably the result of the muscle cell damage promoting both the release of free iron from haem proteins and lipid oxidation products emerged from the rupture of adipocytes (Tokur & Korkmaz, 2007; Standal et al., 2018).

Results of carbonyl content determined from fluorescence microscopy images of protein extracts subjected to CHH-labeling significantly correlated with total carbonyl data obtained by the developed fluorometric microplate assay ($R = 0.957$, $p < 0.05$) and standard ELISA method ($R = 0.911$, $p < 0.05$) based on immunocytochemistry. This shows that the proposed CHH-labeling method can be successfully applied not only for visualization of carbonylated molecules, but also for their quantification. Moreover, the proposed method can allow determination of total carbonyls in insoluble protein fractions, which is not possible in standard ELISA protocol. In addition, the new fluorescence method based on CHH– labeling is much faster (i.e. 7 h versus 18–20 h), less laborious and much cheaper (no antibodies required) compared to ELISA method.

3.4. Determination of protein carbonyls in insoluble protein fractions by the developed fluorometric assay in fluorescence microscopy format

As shown above, the developed fluorometric assay based on CHH-labeling of carbonylated species can be used in fluorescence microscopy format for both visualization and quantification of protein carbonyls. This specificity provides a good opportunity for determination of carbonyls in insoluble protein fractions which cannot be analyzed by ELISA method.

Thus, in order to detect and quantify carbonyls in insoluble protein fractions of biological samples, connective tissue proteins extracted from fish minces were CHH-labeled and visualized by fluorescence microscopy as described in *Materials and Methods*. BSA-standards subjected to CHH-labelling were subjected to fluorescence imaging along with the experimental samples for construction of calibration curve. The results of protein carbonyls in connective tissue proteins of fish minces obtained by the developed fluorometric assay are shown in Fig. 5. The microscopic visualization of carbonylated segments of the connective tissue proteins is displayed in Fig. 4 I-P. The segments of the carbonylated fish tissue fibrils are fluorescent on the dark background of the displayed micrographs. It is clearly seen that fresh fish minces has very low content of carbonyls in both soluble (Fig. 3) and insoluble protein fractions (Figs. 4–5) compared to frozen minces. These data are in agreement with previous research of Standal et al. (2018) investigating protein carbonylation in fish during frozen storage. The negative effect of freezing and subsequent frozen storage on protein oxidation in the analyzed fish minces can be explained by a greater membrane damage compared to fresh fish (Standal et al., 2018). Thus, freezing favors the rupture of the cell membranes of adipocytes, liberating lipid oxidation products that further come in contact with proteins leading to protein carbonylation (Standal et al., 2018).

4. Conclusion

The present study has displayed a novel reliable and sensitive fluorimetric assay based on CHH-labelling of carbonylated species aiming to detect, visualize and quantify protein carbonyls in both soluble and insoluble protein fractions. The described method was tested and validated on fish tissue extracts, but may presumably be applied to other biological samples having protein content of 1 mg/ml or above. The main benefits of the assay are: rapidness, non-invasiveness, highly reproducibility and simplicity as it can be applied in a microplate or fluorescence microscopy format, while being suitable for both relative and absolute quantification. The application of the developed method in a fluorescence microscopy format allows visualization and quantification of carbonylated proteins in insoluble protein fractions. However, for visualization and analyzing of connective tissue extracts, it would be necessary to remove lipid fractions (because they also contain carbonyls) prior to CHH-labelling.

It has been revealed that the developed fluorimetric assay and the standard ELISA method provided similar results of protein carbonyls in different samples, indicating high repeatability and validation of experimental data. However, the fluorescence visualization and subsequent quantification of carbonylated non-soluble protein fraction can only be performed for highly oxidized proteins. Therefore, to solve this challenge, further studies should be performed in this direction.

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