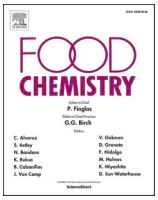
Pro-oxidative activity of trout and bovine hemoglobin during digestion using a static in vitro gastrointestinal model

Food Chemistry 393 (2022) 133356

Contents lists available at ScienceDirect

Food Chemistry

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



Pro-oxidative activity of trout and bovine hemoglobin during digestion using a static *in vitro* gastrointestinal model

Haizhou Wu *, Cecilia Tullberg, Semhar Ghirmai, Ingrid Undeland *

Department of Biology and Biological Engineering-Food and Nutrition Science, Chalmers University of Technology, SE 412 96 Gothenburg, Sweden

ARTICLE INFO

Keywords:
Lipid oxidation
Aldehydes
Heme protein
Hemin release
Protein hydrolysis
Malondialdehyde
4-hydroxy-2-hexenal
4-hydroxy-2-nonenal

ABSTRACT

The degradation of trout and bovine hemoglobin (Hb) and their pro-oxidant activities in washed cod muscle mince (WCM) were studied using simple pH-shifts to simulate gastrointestinal (GI) conditions (pH 7 → 6 → 3 → 7), as well as full static *in vitro* GI digestion. Following gastric acidification to pH 6, metHb formation increased, especially for trout Hb. Subsequent acidification to pH 3 promoted Hb unfolding and partial or complete heme group-loss. During full GI digestion, polypeptide/peptide analyses revealed more extensive Hb-degradation in the gastric than duodenal phase, without any species-differences. When digesting WCM +/-Hb, both Hbs strongly promoted malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE), and 4-hydroxy-2-nonenal (HNE) formation, peaking at the end of the gastric phase. Trout-Hb stimulated MDA and HHE more than bovine Hb in the first gastric phase. Altogether, partially degraded Hb, and/or free hemin -both mammal and fish-derived- stimulated oxidation of PUFA-rich lipids under GI-conditions, especially gastric ones.

1. Introduction

Red meat consumption has received considerable attention because of the risks for e.g. colorectal cancer (CRC), negative environmental effects, and non-sustainable land use issues (Bertolotti, Carfora, & Catellani, 2020). Regarding the former, heme-proteins and their capacity to stimulate lipid oxidation is hypothesized to be one of the risk factors, while these proteins at the same time are nutritionally important since heme iron has significantly higher bioavailability compared with non-heme iron (Buzala, Slomka, & Janicki, 2016). Substituting red meat by fish protein has been found to lower the risks of CRC as well as cardiovascular disease and all-cause mortality, based e.g. on a study that tracked 29,682 men and women for up to 30 years (Zhong et al., 2020). Since fish also contains substantial amounts of heme-proteins, especially in small non-bled dark muscle species as herring, sardines and sprat (Wu, Ghirmai, & Undeland, 2020), the risk for anemia among e.g. young women, can also be circumvented by such a substitution. Mild anemia is otherwise a common side-effect in this consumer group when shifting from a meat containing- to a fully vegan diet.

In red meat, heme iron exists mainly as myoglobin (Mb) while in fish, the main heme-source is hemoglobin (Hb) (Richards, 2010). Reported levels of Mb versus Hb on a heme base are 155 and 77 μmol/kg, respectively, in beef *semimembranosus* muscle, and 382 and 488 μmol/kg, respectively in mackerel (*Scomber scombrus*) dark muscle (Richards & Hultin, 2002). Given the dual role of heme-proteins for human health, it is somewhat surprising that so little research has been conducted to monitor their specific degradation and changes in the heme group during gastrointestinal (GI) digestion. Exceptions are for example Li et al. (2020), who studied the degradation of horse Mb in a static *in vitro* GI digestion model and found that Mb was not susceptible to complete proteolysis, which could have been attributed to its rigid structure. Further, Caron et al. (2016) used low-resolution versus high-resolution mass spectrometry (MS) to identify the degradation products of bovine Hb in an *in vitro* GI system. They identified 75 unique peptides at the end of digestion. Despite these studies, and despite the ongoing dietary protein shift, there is to the best of our knowledge no studies comparing the degradation of mammalian and fish heme-proteins during GI digestion.

Fish muscle is usually more susceptible to lipid oxidation during storage and process compared with red meat since it contains highly pro-oxidative Hb (Aranda et al., 2009) and high amount of polyunsaturated fatty acids (PUFA) (Wu, Abdollahi, & Undeland, 2021). However, as stated above, heme-mediated oxidation in red meat is still described as one of the potential routes to meat-induced CRC, which is linked to the ability of this reaction to continue also during GI digestion and generate reactive aldehydes such as e.g. malondialdehyde (MDA) and 4-hydroxy-

* Corresponding authors.
E-mail addresses: haizhou@chalmers.se (H. Wu), undeland@chalmers.se (I. Undeland).

https://doi.org/10.1016/j.foodchem.2022.133356
Received 25 January 2022; Received in revised form 11 May 2022; Accepted 29 May 2022
Available online 31 May 2022

2-nonenal (HNE) (Sicard, Mirade, Portanguen, Clerjon, & Kondjoyan, 2018). We (Larsson, 2016) and others (Steppeler, Haugen, Rødbotten, & Kirkhus, 2016) have reported that MDA can also form during *in vitro* digestion of fish muscle and marine oils. However, in these studies, MDA co-appeared with 4-hydroxy-2-hexenal (HHE) rather than HNE (Steppeler, Haugen, Rødbotten, & Kirkhus, 2016; Tullberg & Undeland, 2021). This is since HHE is specifically connected to oxidation of n-3 PUFAs, while HNE is reported to be the main product from n-6 PUFA oxidation (Pryor and Porter, 1990). MDA, on the other hand, can be derived from a wide range of PUFAs and is a commonly chosen as an unspecific marker for lipid oxidation (Larsson, 2016). MDA, HNE and HHE have all been ascribed potential cytotoxic and genotoxic effects, with HNE having the lowest median lethal concentration (LC_{50}) (Cerbone et al., 2007). From our studies comprising dynamic GI static *in vitro* digestion of cod liver oil (Larsson, 2016), it was found that cod Hb could promote oxidation, generating MDA, HHE and HNE. However, the exact pro-oxidative mechanism of Hb has not been clarified, and also not whether there are differences between Hbs coming from mammalian versus fish sources.

The aims of this study were to: (i) investigate the degradation of trout and bovine Hb during the pH-cycle taking place in the GI-digestion, (ii) compare the pro-oxidative activity of these two Hbs during a full static *in vitro* digestion when added to a washed cod muscle matrix, and (iii) assess the potential mechanisms behind Hb-meditated lipid oxidation during static *in vitro* GI digestion by combing the outcome of (i) and (ii).

2. Materials and methods

2.1. Chemicals

Pancreatic α-amylase (Sigma-A3176), porcine gastric mucosa pepsin (Sigma-P7000), *Rhizopus oryzae* lipase (Sigma-80612), porcine pancreas pancreatin (Sigma-P1750), and porcine bile extract (Sigma-B8631) were obtained from Sigma Aldrich (Saint Louis, MO, USA). HHE and HNE were obtained from Cayman (Ann Arbor, MI, USA). All other chemicals and solvents used in digestions as well as in analyses of Hb and lipid oxidation were of at least analytical grade.

2.2. Preparation of hemolysate and hemoglobin quantification

Trout (*Oncorhynchus mykiss*) kept in tanks at Gothenburg University was bled as described by Ghirmai et al., (2020) and fresh bovine blood was provided by a local slaughterhouse (KLS Ugglarps AB, Senåsa, Sweden). To avoid coagulation, 4 volumes of blood were mixed with 1 vol of anticoagulant (150 mM NaCl and 120 units/mL sodium heparin). Trout and bovine hemolysates were then prepared as described by Wu et al. (2021). The blood samples were washed four times with 1 mM Tris, pH 8, 0.9% NaCl to obtain red blood cells. Hemolysates were obtained by lysing the red blood cells with 1 mM Tris, pH 8. The extinction coefficient of 132 mM^{-1} cm^{-1} at 415 nm was used to measure the concentration of oxyHb on a heme basis (Wu, Xiao, Yin, Zhang, & Richards, 2021).

2.3. Preparation of washed cod muscle

Fresh cod (*Gadus morhua*) fillets were obtained from Landala Fisk (Göteborg, Sweden). Washed cod muscle was prepared as described by Richards and Hultin (2002). The connective tissue and dark muscle were removed manually, and the white muscle was ground by a kitchen grinder (Model KSM90, KitchenAid, St Joseph, MI, USA). Fish mince was washed once with 3 volumes (w/w) of cold (4 °C) Milli-Q water and twice in 3 volumes of cold 50 mM phosphate buffer (pH 6.8). In the last wash, mince and solution were homogenized for 1 min using a polytron at speed 3 (T18 basic, IKA Works, Wilmington, NC). The washing buffer was removed by centrifugation at 15,000g at 4 °C for 25 min. The pellet was collected as washed cod muscle (WCM) and packed in Polynova plastic bags (89 mm × 114 mm, 50 μm). The air in the bag was manually removed by flattening the packages, and the samples were stored at −80 °C.

2.4. Subjection of hemoglobin to a pH-cycle simulating human GI-conditions

To evaluate the effect of the pH-values used in simulated GI digestions on conformation, autoxidation and degradation of Hb, trout and bovine hemolysates were mixed with electrolyte solution (Supplementary material Table S1) to a final concentration of 10 μM Hb on a heme basis. The incubation time, pH, and temperature applied were according to the INFOGEST protocol (Minekus et al., 2014). Seven sampling points were selected during the pH-cycle (see Fig. 1).

2.5. Subjection of hemoglobin (+/− washed cod mince) to a full gastrointestinal in vitro digestion

Before the digestion trial, the activity of all digestive enzymes (α-amylase, lipase, pepsin, and trypsin) and bile-salt concentration were analyzed by the method of Minekus, et al. (2014). The following eight different samples were then subjected to *in vitro* GI digestion according to a modified version of the first INFOGEST protocol (Minekus, et al., 2014) published by Tullberg et al. (2019): (1) Milli-Q water (blank); (2) trout hemolysate (40 μM Hb); (3) trout hemolysate (1 mM Hb); (4) bovine hemolysate (40 μM Hb); (5) bovine hemolysate (1 mM Hb); (6) WCM; (7) WCM + trout hemolysate (40 μM Hb); (8) WCM + bovine hemolysate(40 μM Hb). The preparation of SSF, SGF and SIF and addition of enzymes, bile salts, and Ca^{2+} solution are shown in Supplementary material Table S1. The pH, dilution, and sampling points are presented in Fig. 1. In brief, 7 g (for hemolysate-fortified WCM samples) or 7 mL (for blank and hemolysate-based samples) were mixed with 7 mL of simulated salivary fluid (SSF). Then, the samples were mixed with 10.5 mL simulated gastric fluid (SGF) and pH was adjusted to pH 6.0 for 60 min of digestion and then the pH was adjusted to pH 3.0 for another 60 min of digestion. Next, the samples were mixed with 7 mL simulated intestinal fluid (SIF) and the pH was adjusted to 7 for 90 min digestion. After each addition of digestive fluid, samples were flushed with N_2 gas (15 s) to reduce oxygen content. Samples were immediately snap frozen in liquid nitrogen to stop the enzyme activity. However, for samples with high Hb concentration (1 mM), which were used for high-performance size-exclusion chromatography (HP-*SEC*) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), they were mixed with 1 M NaOH 1:1 (v/v) to stop enzyme activity (Tullberg, Vegarud, & Undeland, 2019), and then snap frozen in liquid nitrogen.

2.6. Scanning of hemoglobin-containing samples for determination of e.g. metHb

Samples from section 2.4, subjected only to the pH-cycle used in *in vitro* digestion, were clarified by centrifugation (16,000 g for 3 min) prior to conformational and metHb-determination studies by UV/VIS-spectroscopy (Kristinsson, 2002). Supernatants were scanned from 350 to 700 nm with a spectrophotometer (Cary 60 UV–vis, Agilent technologies, Santa Clara, CA, USA). For metHb-determination, the absorbance at 560, 576, 630 and 700 nm was recorded and the equations of Benesch, Benesch, and Yung (1973) were applied. When the pH was shifted to 3, the Hb was however unfolded, and the equation could not be used to calculate the metHb percent. Thus, only samples at pH 7 and 6 were analyzed by this method.

2.7. Analyses of hemoglobin content and hemin loss

For samples from section 2.4 and 2.5, Hb content was measured as described by Drabkin and Austin (1935). A 20 μL digestive sample was mixed with 980 μL Drabkin's Reagent and incubated for at least 15 min

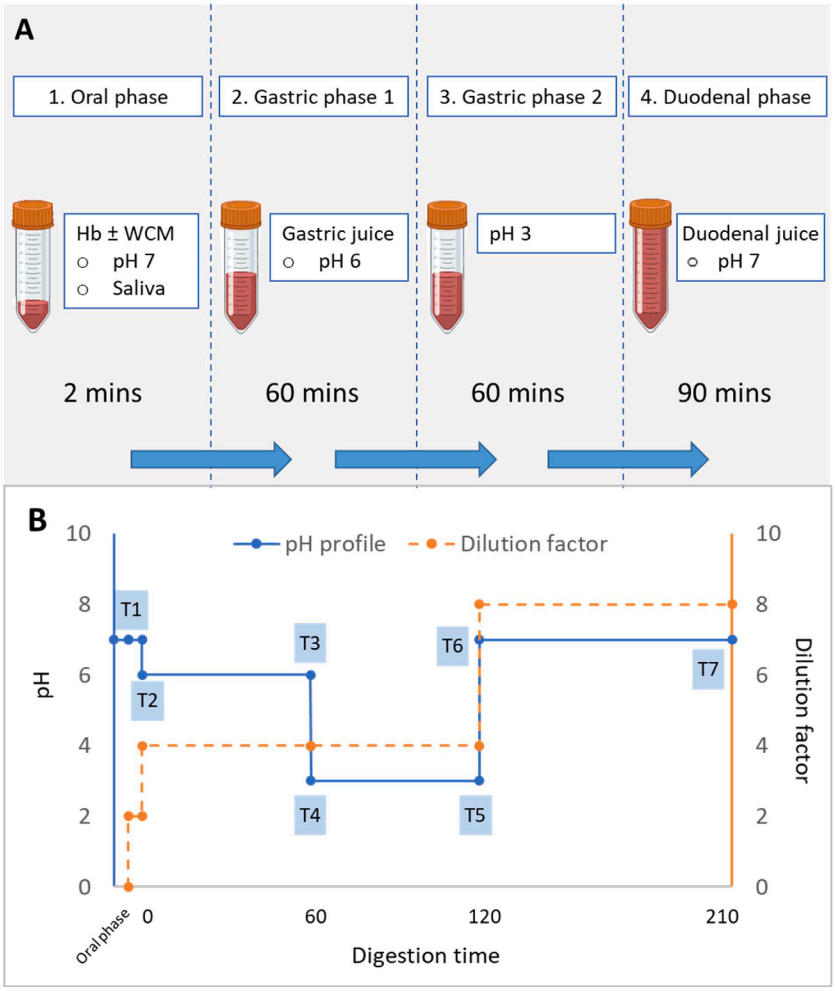


Fig. 1. Flow chart of the static *in vitro* digestion protocol (A) and changes in pH as well as initial meal dilution factor over time during the *in vitro* digestion (B). Sampling times are marked as T1-T7.

at room temperature. Then the absorbance of the mixture was measured at 540 nm after centrifugation of the samples at 16,000 g, 2 min. A standard curve was prepared with 0.15–77 μM concentrations of bovine Hb in 50 mM Tris-HCl, pH 8.6.

Loss of free hemin from metHb was measured by subjecting digests from section 2.5 to solid phase extraction (SPE) with C18 cartridges (Biotage, Uppsala, Sweden) as described by Maestre, Pazos, and Medina (2009). Briefly, 2 mL of a water/methanol (1:1) was used for precondition to cartridges and then 1 mL of Hb sample was passed through. Later, 4 mL of water was used to wash cartridges and cartridges were dried for 15 min by passing through a flow of air by action of a vacuum pump. Lastly, free hemin was eluted with 1 mL of methanol, and the absorbance peak of hemin in the visible spectrum (350–450 nm) was acquired.

2.8. High-performance size-exclusion chromatography (HP-SEC) of high Hb digests

Digests from section 2.5 with the high Hb concentration (1 mM) were centrifuged for 3 min at 16,000 g, and supernatants were collected for HP-*SEC* analysis (HP-*SEC*; Dionex HPLC, Dionex GmbH, Idstein, Germany) with an Agilent Bio *SEC*-5 column (5 μm particle size, 150 Å pore size) to determine their apparent molecular weight. Based on the protein concentration of supernatants as analyzed according to Lowry et al. (1951), all samples were diluted to a final protein concentration of 2.6 mg/mL prior to injection of 10 μL samples into the HP-*SEC* system. A mixture of protein standards (1–670 kDa) was used to prepare a calibration curve (Supplementary material Fig. S1). The mobile phase was fresh sodium phosphate buffer (0.1 M, pH 7.5) with a flow of 0.2 mL/min, and the absorbance at 214 nm was recorded for 30 min for each sample. The relative proportion of each peak was calculated based on peak areas towards standards.

2.9. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

To monitor Hb degradation during the full *in vitro* digestion, and estimate the molecular weight of the polypeptide chains, the digests (2.6 mg/mL) from section 2.5 were subjected to denaturing SDS-PAGE as described by Abdollahi et al. (2019).

2.10. Analysis of MDA, HHE and HNE

The HHE, HNE, and MDA concentrations of digests from the full *in vitro* digestion (section 2.5) were measured by 2,4-dinitrophenylhydrazine (Brady's reagent) derivatization of aldehydes followed by LC/APCI-MS analysis (Tullberg et al., 2016). The standard curve and chromatograms are shown in Supplementary material Fig. S2.

2.11. Statistical analysis

All statistical analysis was conducted with SPSS software (SPSS

Statistics Version 22, IBM Inc., Chicago, IL, USA). The results are reported as mean ± standard deviation from at least two replicate digestions ($n \geq 2$). Duncan's multiple range test was used to compare the means. Variance (ANOVA) was used to analyze the significant differences between treatments and/or time points. Differences are regarded as significant when $p < 0.05$.

3. Results

3.1. Content, conformation, and auto-oxidation of hemoglobin as a function of pH

During digestion, the pH undergoes extreme changes, from the oral pH 7, to gastric pH 6 and 3, and back to intestinal pH 7 (Minekus, et al., 2014). Based on the sensitivity of Hb towards pH-changes, it was of interest to investigate how the pH-cycle appearing during digestion might affect the content, conformation and auto-oxidation of Hb. This assessment could provide useful information regarding the degradation and associated pro-oxidant ability of Hb in the digestive tract. The visible spectrum of Hb provides information about the heme-globin linkage, oxidation of heme iron, and ligand binding state of the protein (Kristinsson, 2002).

Fig. 2 shows the spectra of trout and bovine Hb as a function of time and pH. At the initial point (0 min), both trout and bovine Hb showed a Soret band absorption maximum at ~415 nm and two sharp peaks near 576 and 540 nm forming a deep valley; a spectrum which was indicative of a high initial content of oxyHb compared to other forms, such as deoxy- or metHb (Wu, Sajib, & Undeland, 2021). Following pH adjustment to 6 and incubation for 1 h (simulated gastric phase 1), the Soret band of the trout Hb shifted to 405 nm, whereas the bovine Hb shifted only slightly. The shift indicted that trout Hb more rapidly was converted into metHb compared with bovine Hb. The results in Fig. 3

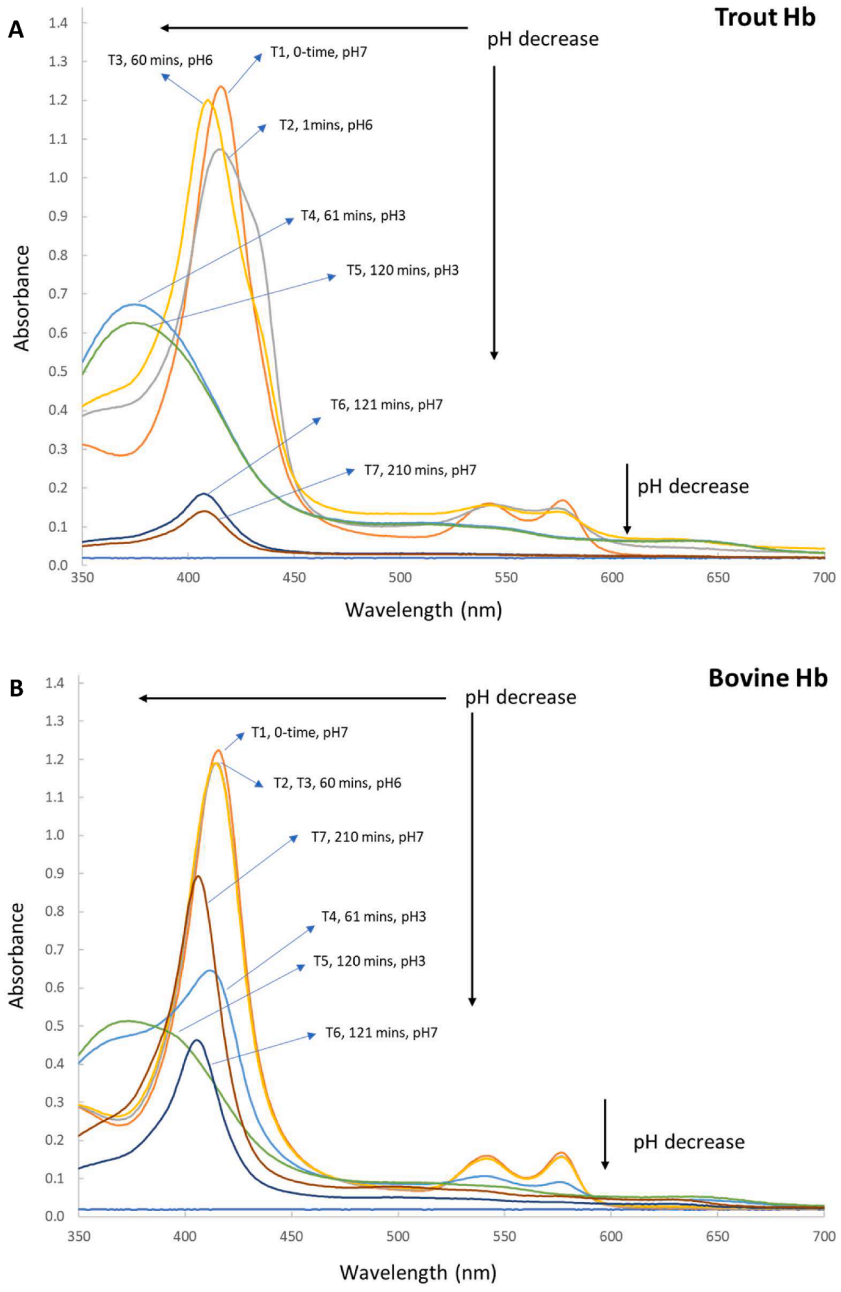


Fig. 2. UV/VIS spectroscopy analysis of trout (A) and bovine (B) Hb diluted in electrolyte under a pH-cycle and at 37 °C simulating human GI-conditions (i.e., a static digestion protocol without enzymes).

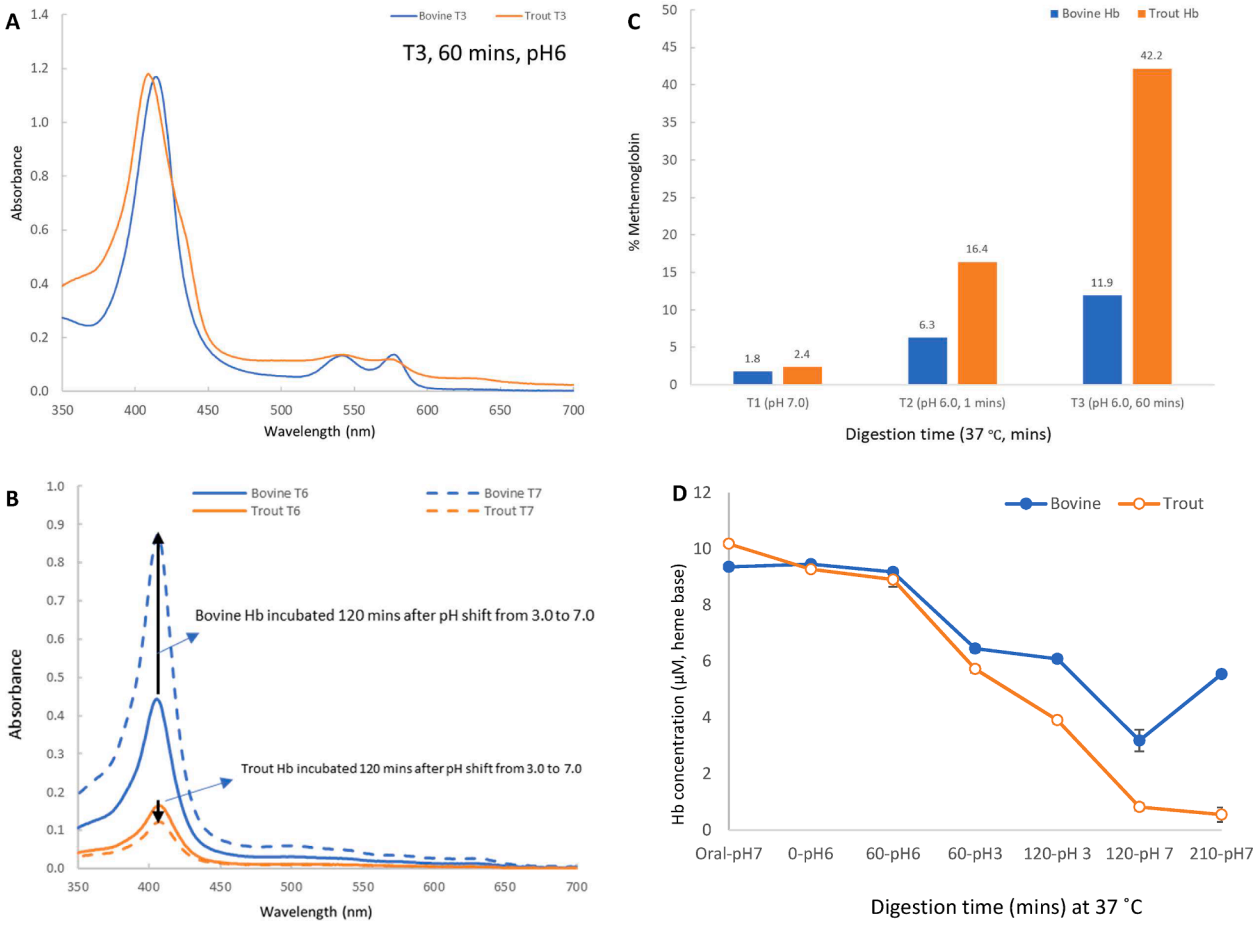


Fig. 3. UV/VIS spectra of trout and bovine Hb at 60 mins (A), and 120 mins (B), as well as the metHb percentage change (C), the Hb contents change (D) during pH-cycle and at 37 °C simulating human GI-conditions (i.e., a static digestion protocol without enzymes).

confirmed this conclusion, namely, 42.2% of the trout Hb was metHb compared with 11.9% of the bovine Hb after 60 min. Furthermore, when the pH was further adjusted to 3, and samples were incubated for an additional hour (simulated gastric phase 2), the Soret bands were blue shifted (~380 nm) (Fig. 2).

Although trout Hb had a similar blue-shifted spectra as bovine Hb when incubated for one hour at pH 3, the two Hbs showed a significant difference ($p < 0.05$) in Hb recovery, right after the pH was adjusted to 7 (duodenal phase) (Fig. 3B and 3D). Fig. 3B shows the significantly ($p < 0.05$) higher Soret band absorption of bovine Hb compared with trout Hb after 90 min incubation at pH 7. This observation indicated that bovine Hb could properly refold, but trout Hb could not refold, which may explain why the concentration of properly folded bovine Hb significantly ($p < 0.05$) increased from 3.18 to 5.55 μM during 90 min subjection to duodenal pH (7), while trout Hb declined from 0.82 to 0.55 μM in this period (Fig. 3D).

3.2. Hb contents and hemin release during full in vitro GI digestion

During full *in vitro* GI digestion, the reaction environment of Hb is complex, which renders analysis of its different forms difficult. For this reason, a relatively high concentration of Hb was used (1 mM) to capture the denaturation and degradation of Hb against the high protein background provided e.g., by digestive enzymes. Fig. 4A shows the concentration of intact trout and bovine Hb in the samples taken during the entire GI digestion process. After the Hb was digested for 1 h at pH 6 in the first gastric phase, the trout Hb content dropped significantly ($p < 0.05$) from 19.0 to 10.6 μM, whereas the content of bovine Hb did not significantly change. However, contents of both trout and bovine Hb significantly ($p < 0.05$) decreased and arrived at almost the same concentration after the pH was changed from 6 to 3 (Fig. 4A). During the second gastric phase and the duodenal phase, levels of both trout and bovine Hb were continuously diminished and were close to 0 by the end of the duodenal phase.

The heme group in Hb has a key role for its pro-oxidant activity which in turn is linked to the form of heme-free or globin-bound (Richards, 2010). For example, Wu, Yin, Zhang, and Richards (2017) reported that released hemin was the primary promoter of lipid oxidation in washed fish muscle mince. Based on this, the free hemin concentration derived from trout and bovine Hb during the digestion process was monitored. Fig. 4B shows that a significantly higher concentration of free hemin was present in digests taken from trout Hb during the first gastric phase (1 h digestion at pH 6) compared with bovine Hb digests. When the pH was adjusted to 3, both sample types rapidly increased in free hemin and reached the same concentration (Fig. 4B). When re-adjusting the pH to 7, free hemin significantly declined again, then staying low throughout the duodenal phase. This could reflect the tendency of hemin to aggregate in aqueous solutions at neutral pH (Glahn, Wortley, South, & Miller, 2002).

3.3. Polypeptide pattern and molecular weight of Hb during full in vitro GI digestion

Hb can be hydrolyzed by several GI digestive enzymes, primarily pepsin, trypsin, chymotrypsin and carboxypolypeptidases. To map the gradual proteolytic degradation of trout and bovine Hb, HP-*SEC* and denaturing SDS-PAGE was applied to the digests sampled during the full GI digestion. Fig. 5A and B shows the *SEC*-results of trout and bovine Hb,

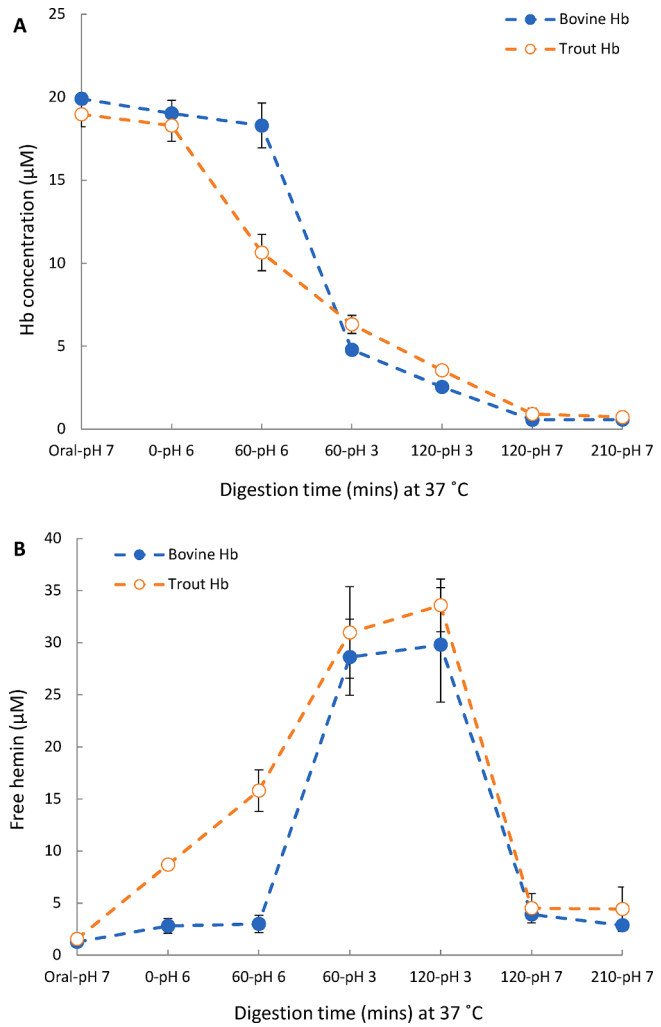


Fig. 4. The content of Hb (A) and free hemin (B) during full *in vitro* gastrointestinal digestion of trout and bovine Hb. Data show average values from two replicate digestions ± SD (n = 2).

respectively, at four important time points; start of digestion −0 min, end of first gastric phase −60 min, end of second gastric phase −120 min, and end of duodenal phase −210 min. At start of the digestion, only one peak was observed for both trout and bovine Hb, at ~ 64 kDa. This single peak indicated high purity of the Hb samples, which is confirmed in Fig. 5C, revealing that the 64 kDa Hb-peak contributed to > 98% of the total protein/(poly)peptide area. By the end of gastric phase 1, the area of the 64 kDa peak had decreased, and two new peaks appeared at 3.9 kDa and 1.0 kDa (Fig. 5A and 5B). During the second gastric phase and the duodenal phase, the peak at 64 kDa disappeared completely while the peaks at 3.9 kDa and 1.0 kDa increased further. Fig. 5C shows that at the end of the gastric phase, 65.8% and 77.1% of the total peak area was made up of peptides<18 kDa for trout and bovine Hb, respectively, while at the end of the duodenal phase, the two Hbs had almost the same polypeptide pattern, with 80% of the peptides being in the range 1.3–18 kDa, and 20% <1.3 kDa.

Hb is a tetramer composed of two α- and two β-polypeptides that each resemble myoglobin Mb (16 kDa). The Hb monomers at 16 kDa were clearly visible in the SDS-PAGE results at start of the digestion (Fig. 5D). With increasing digestion time, the band at 16 kDa however gradually weakened and the lower molecular weight (LMW) bands increased. For trout Hb, new bands appeared at 6.5 and 5 kDa after first gastric phase −60 min at pH 6.0 (lane 3) while after the second gastric phase −120 min at pH 3.0, the band below 6 kDa had increased further. For bovine Hb, new bands appeared around 14.2 kDa and 6.5 kDa after the first gastric phase −60 min at pH 6.0 (lane 8), which were converted into a thick band at 3.5–6 kDa after second gastric phase −120 min at pH 3.0 (lane 9). At the end of the second gastric phase, the band at 16 kDa had disappeared almost completely for both trout and bovine Hb (lanes 4 and 9) in favor of LMW-bands, supporting the *SEC*-data. At the end of the duodenal phase (lanes 5 and 10), no notable bands were found at all, revealing formation of amino acids and peptides which were too small to stay on the gel.

3.4. Lipid oxidation of cod muscle with or without Hb during the full in vitro GI digestion

Certain aldehydes formed as secondary lipid oxidation products are highly reactive and can explain the potential toxic consequences of lipid oxidation (Cerbone, et al., 2007). The aldehydes HHE, HNE and MDA are of special interest when studying lipid oxidation in marine foods due to their abundance in n-3 and n-6 PUFA, especially the former. These aldehydes were therefore followed in washed cod muscle mince, with or without added Hb, during the entire *in vitro* digestion process. Fig. 6A shows that washed cod muscle without Hb increased in MDA content from 2.4 to 14.3 during the two gastric phases. However, the MDA content in washed cod muscle with addition of 40 μM Hb increased more rapidly to a maximum value of 30.5 μM at 120 min. Trout Hb provided significantly ($p < 0.05$) higher MDA-levels compared with bovine Hb, which was evident already from start of the digestion (Fig. 6A). HHE and HNE developed according to a similar pattern as that of MDA, but reached significantly lower total maximum levels and peaked at 1.9 and 0.1 μM, respectively, after 120 min (Fig. 6B and 6C). As for MDA, trout Hb promoted significantly ($p < 0.05$) more HHE than bovine Hb in the gastric phases, even if starting values for HHE were similar. In the duodenal phase, the development rate for the three aldehydes levelled off and in Hb-containing samples, significant ($p < 0.05$) declines were seen (Fig. 6).

4. Discussion

4.1. Effect of pH alone on solubility, content, conformation and auto-oxidation of hemoglobin

Auto-oxidation of oxy/deoxyHb into metHb is an important factor determining the rate of Hb-mediated lipid oxidation in muscle matrices since metHb may possess a greater pro-oxidant activity compared to oxyHb (Wu, Yin, Zhang, & Richards, 2017). MetHb formation from both trout and bovine Hb was promoted when reducing pH from 7 to 6 in absence of enzymes (Fig. 3A and 3C) may therefore be very critical from a lipid oxidation perspective. We previously found that the greater catalytic activities of trout Hb at pH 6 compared with at pH 7.2 in a washed cod muscle mince corresponded to greater metHb formation (Undeland, Kristinsson, & Hultin, 2004). The rapid auto-oxidation of Hb at low pH is described to arise from the ability of protons (H^+) to enter the heme crevice and protonate liganded O_2 (Richards, 2010). Richards et al. (2009) reported that a pH reduction from 8.0 to 6.3 caused structural changes in perch Hb, and they observed a larger channel for solvents into the heme crevice, stimulating induction of metHb formation. Trout Hb showed a greater metHb formation after one hour at pH 6 compared with bovine Hb (42.2 % versus 11.9%), which could be attributed to the Bohr effect. This phenomenon, which involves a shift in the oxygen dissociation curve with pH and CO_2-pressure, occurs in fish Hbs, but only slightly in mammalian Hb (Aranda, et al., 2009).

At pH 3, in absence of GI-enzymes, both trout and bovine Hb showed spectra which were significantly blue-shifted (Fig. 2A and 2B), indicating that the heme group was likely partly or completely lost, because the heme-globin linkage and the heme group may be converted to four-coordinate, low-spin species at pH 3 (Kristinsson, 2002). This conversion could be attributed to the partially unfolded globin structure and heme

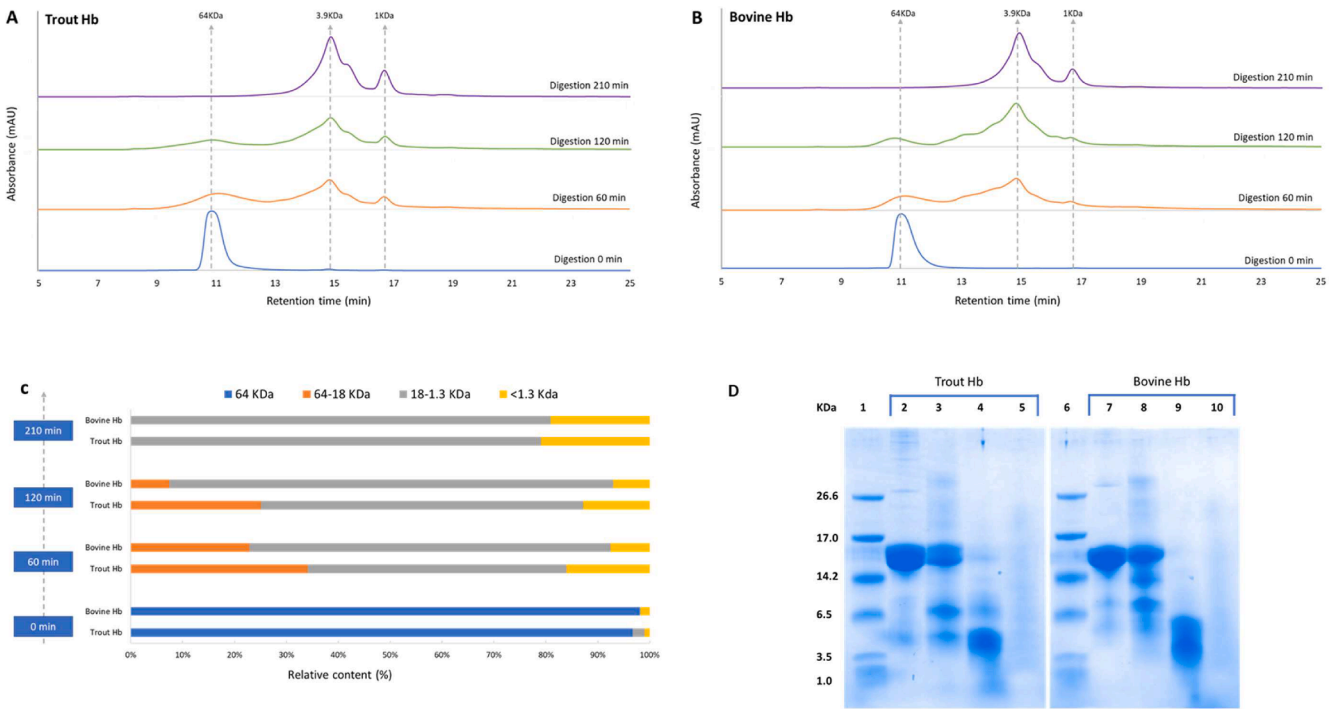


Fig. 5. Changes in molecular weight distribution of trout and bovine Hb during full *in vitro* gastrointestinal digestion as measuerd by HP-*SEC* and SDS-PAGE; (A) *SEC* chromatogram for trout Hb, (B) *SEC* chromatogram for bovine Hb, (C) relative content of differently sized peptides, (D) SDS-PAGE gels. Lanes show: 1- molecular weight marker; 2- Trout Hb, 0 mins; 3- Trout Hb, 60 mins digestion; 4- Trout Hb, 120 mins digestion; 5- Trout Hb, 210 mins digestion; 6- molecular weight marker; 7- Bovine Hb, 0 mins; 8- Bovine Hb, 60 mins digestion; 9- Bovine Hb, 120 mins digestion; 10- bovine Hb, 210 mins digestion.

group exposure seen at pH 3 (Kristinsson & Hultin, 2004). Similarly, Kristinsson (2002) reported that flounder Hb formed various stable structures at pH 4–5.5, in which the heme was likely still bound to the proximal histidine but slightly displaced from the heme crevice. At pH 3.5, a mixture of displaced heme, unbound or bound to the proximal histidine, was seen (Kristinsson & Hultin, 2004). Unfolding of the distal heme pocket at low pH could increase the pro-oxidant ability of Hb. At low pH, there can be an irreversible loss of oxygen binding with subsequent autoxidation of heme–iron into metHb (Aranda, et al., 2009). This change could in turn promote loss of the heme group because oxygen binding has a large effect on the proximal histidine – heme interactions (Aranda, et al., 2009).

We found that the pH-change from 6 to 3 in absence of enzymes reduced the content of both trout and bovine Hb (Fig. 3D) which was likely due to Hb-unfolding. This hypothesis is supported by the results from Kristinsson (2002) who used intrinsic tryptophan fluorescence to monitor Hb unfolding during a change in pH from 7.5 to 1.5. Unfolding was detected when the pH was lower than 5.5, and was largest as the pH dropped from 4.0 to 3.0 (Kristinsson, 2002). That the content of bovine Hb increased again in the pH-span 6.0–7.0 when re-adjusting the pH from 3.0 to 7.0 (Fig. 3D), indicates refolding of the Hb-structure. This phenomenon may however not occur in presence of the proteolytic enzymes which are part of full GI-digestion. Also, it should be stressed that the actual time period during which a Hb-containing meal is exposed to pH 3 during gastric digestion depends on the buffering capacity of the meal ingested. As described by Sams et al. (2016), it appears that gastric pH remains rather high during gastric digestion and decreases at a slow rate until 50–60% of the meal is emptied from the stomach. With liquid and solid test meals, gastric pH at 50% gastric emptying was still found at mean values of of 5.5 and 4.6, respectively (Sams, Paume, Giallo, & Carrière, 2016).

4.2. Trout and bovine hemoglobin degradation during full in vitro GI-digestion

Both *SEC* and SDS-PAGE (Fig. 5) showed that more pronounced proteolytic degradation of Hb occurred in the two gastric phases compared with the duodenal phase; there was no intact Hb at the end of the second gastric phase (Fig. 5). These findings mirror the *in vivo* digestion in humans, in which proteins are digested mainly in the stomach. Similarly, by using the harmonized INFOGEST static protocol (Minekus, et al., 2014), Sousa et al. (2020) reported that whey protein, collagen, and zein were mainly hydrolyzed in the gastric phase. The gastric proteolytic degradation of Hb is primarily attributed to pepsin, which increases its proteolytic activity as the pH decreases from 6 to 3 (Sicard, Mirade, Portanguen, Clerjon, & Kondjoyan, 2018). When the digests enter the duodenal phase, trypsin and chymotrypsin are the main enzymes continuing the hydrolysis of polypeptide fragments into still smaller fragments or free amino acids. Based on the *SEC* results (Fig. 5A-C), the largest peptide peak was found at 3.9 kDa, which indicated that some polypeptides from Hb were resistant to gastric and duodenal digestion. This resistance to digestion was similar to that found by Li, et al. (2020), who reported that Mb did not completely degrade under static *in vitro* GI digestion, because of its rigid structure.

When the pH was adjusted from 7 to 6 in presence of digestive enzymes, the free heme content derived from trout Hb was greater than that from bovine Hb, which was stable and did not yield free heme (Fig. 4B). Aranda, et al. (2009) found that trout Hb released hemin 26-fold faster than bovine Hb at pH 6.3 and reported on structural and amino acid differences around the heme crevice between fish and mammalian Hb. For example, the lysine at site E10 in bovine Hb was described to form favorable electrostatic and hydrogen bond interactions with the heme 7-propionate group, whereas the smaller threonine at site E10 in trout Hb was described to not interact with the heme (Aranda, et al., 2009). Richards (2010) also reported that hydration of the proximal histidine in mammalian and fish Hb decreased hemin affinity. Another reason why trout Hb released hemin faster than

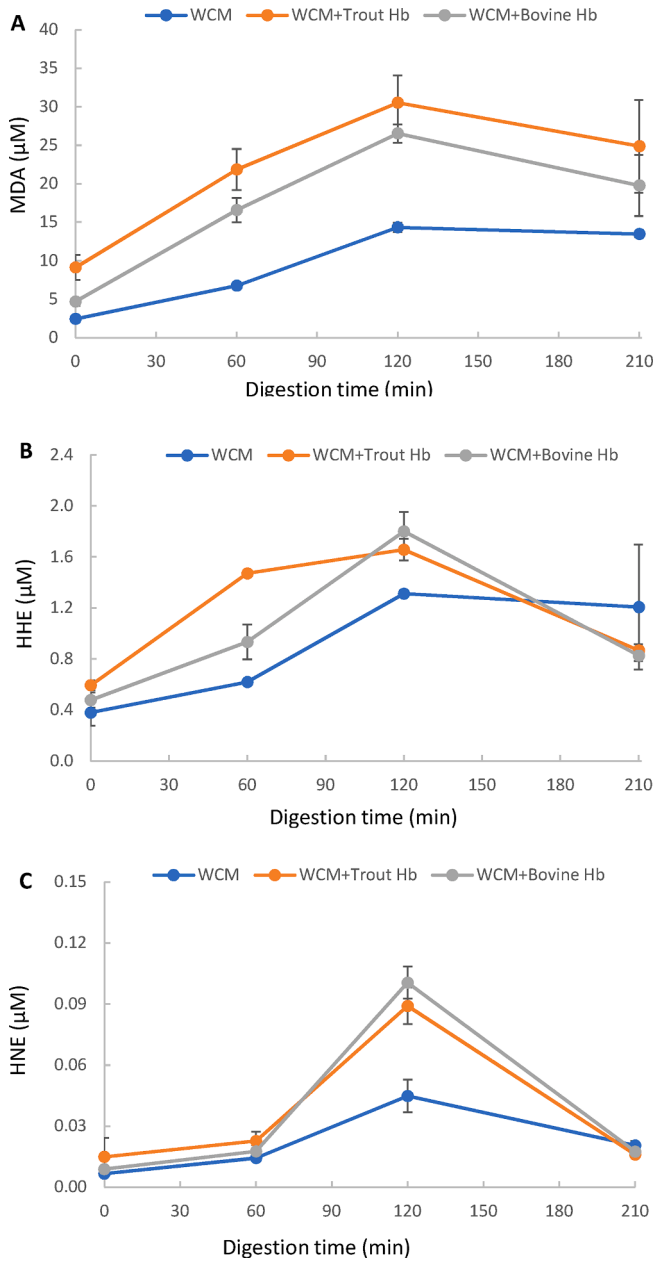


Fig. 6. Formation of MDA, HHE and HNE in washed cod muscle mince with or without Hb during full *in vitro* gastrointestinal digestion. Data show average values from two replicate digestions ± SD (n = 2).

bovine Hb may be attributed to the difference in redox states of trout and bovine Hb. The porphyrin moiety in metMb and metHb is more weakly anchored (60-fold less) in the globin protein compared with the reduced forms (Tang, Kalsbeck, Olson, & Bocian, 1998), and as shown in Fig. 3C, trout Hb generated more metHb compared with bovine Hb, 42.2% versus 11.9%, in absence of proteolytic enzymes.

While we observed extensive hydrolysis of bovine Hb during the first gastric phase (Fig. 5), the free hemin content did not increase (Fig. 4B). This could indicate that the heme group was retained in the polypeptide fragments of the partly hydrolyzed bovine Hb. That hemin was not released could be explained by the specific structure of bovine Hb; particularly the environment around the heme group. While the globin chains of Hb form a compact hydrophilic globular conformation, the heme group is embedded in a hydrophobic pocket of the globular protein (Richards, Aranda IV, He, & Phillips Jr, 2009). We hypothesize that pepsin only partly hydrolyzed the hydrophilic surface of the bovine globin chain and did not affect the structure of the hydrophobic heme crevice. However, for trout Hb, we could not confirm whether pepsin hydrolysis promoted the hemin release seen in the first gastric phase or if it was due e.g. to the pronounced Bohr effect.

When pH was changed from 6 (gastric phase 1) to 3 (gastric phase 2), both trout and bovine Hb provided a rapid increase in hemin concentration (Fig. 4B). This process was completed during < 1 min, thus we suggest that it was not pepsin hydrolysis that contributed to such a large hemin release from Hb, rather, we hypothesize that pH was a more important factor for hemin release. This matches the findings seen during the first gastric phase, wherein pepsin hydrolysis did not contribute to hemin release from bovine Hb. That pH is so critical for hemin release is due to unfolding of the Hb at pH 3 with subsequent disruption of the proximal histidine-heme interactions, releasing hemin (Kristinsson & Hultin, 2004).

In the beginning of the duodenal phase where the pH changed from 3 to 7, there was a clear drop in free hemin content for both trout and bovine Hb in<1 min (Fig. 4B). This could be attributed to hemin aggregates forming when going from pH 3 to neutral pH (7). Glahn, Wortley, South, and Miller (2002) reported that purified hemin formed high molecular weight aggregates at neutral pH which were poorly bioavailable. It is likely that these aggregated ended up in the non-soluble phase of the digest and therefore were not detectable with the SPE-based method applied to quantify heme-loss.

4.3. Lipid oxidation during full in vitro GI digestion of washed cod muscle mince in the presence and absence of hemoglobin

During digestion of washed cod muscle without added Hb, MDA, HHE and HNE increased up to 120 min after which the levels ceased or declined (Fig. 6). This aldehyde formation was likely caused by increased solubility of low molecular weight (LMW) trace elements such as iron at low pH, which in the reduced state could stimulate the Fenton reaction (Halliwell, Zhao, & Whiteman, 2000). Also, lipid oxidation was likely stimulated by the elevated temperature and the combined proteolytic and lipolytic action in the stomach, increasing the surface area of the mince.

The addition of trout and bovine Hb to the washed cod muscle however significantly increased lipid oxidation in the gastric phase, based on the production of MDA, HHE and HNE (Fig. 6). Our lipid oxidation results agreed with those of Larsson (2016), who found that addition of cod Hb (11.5 μM) to cod liver oil produced a strong pro-oxidative effect and resulted in increased TBARS formation during the gastric phase of a static *in vitro* digestion. Similarly, cod Hb showed a strong pro-oxidative activity to cod liver oil in a dynamic *in vitro* digestion model (Larsson, Tullberg, Alminger, Havenaar, & Undeland, 2016). Furthermore, in our previous study comprising digestive fluids aspirated from humans, trace levels of Hb from these aspirated fluids may have contributed to the documented lipid oxidation taking place during *in vitro* digestion of cod liver oil (Tullberg, et al., 2016).

Hb-mediated lipid oxidation is a major problem during storage and process of fresh fish and meat, primarily resulting from hemin release triggered e.g. by *post mortem* reductions in pH (Wu, Yin, Zhang, & Richards, 2017). However, the exact mechanism by which Hb promotes lipid oxidation during the digestion processes has not yet been explained. On basis of the current study, we speculate whether full or partial hemin release from Hb as a result of acidifications and/or proteolysis is a key step behind the increased lipid oxidation of washed cod muscle during the gastric phase. As shown here, pH decreases from 7 to 6 to 3 rapidly auto-oxidized Hb, particularly when derived from fish. At the same time, pH 3 caused Hb-unfolding and heme group exposure. These two phenomena would both facilitate hemin release from Hb, and the released nonpolar hemin could partition into nonpolar membrane bilayers stimulating lipid oxidation (Kristinsson & Hultin, 2004). This hypothesis could also explain the lower development of lipid oxidation in the duodenal phase, which could derive from a lower, or even no, pro-

oxidative activity of the potential high molecular weight hemin aggregates forming when the pH was adjusted from 3 to 7. That the levels of MDA, HHE and HNE even decreased in the duodenal phase is likely a result of the highly reactive aldehydes towards peptides, forming Schiff bases or Michael adducts (Larsson, 2016).

In a study by Carlsen and Skibsted (2004), metMb which was mildly hydrolyzed by pepsin was found to have a strongly enhanced pro-oxidative effect towards an oil-in-water (O/W) methyl linoleate emulsion compared to native metMb. More pronounced proteolysis of metMb at lower pH values near the optimum for pepsin did not result in a similar enhancement of pro-oxidative activity. The mildly proteolyzed metMb had spectral characteristics showing a relative stabilization of the iron(II) state. It was therefore suggested that the increased prooxidative effect was due to radicals formed by cleavage of lipid peroxides by iron(II)/ iron(III) cycling of a heme pigment with affinity for the oil-in-water interface. At the same time, pepsin-induced proteolysis at pH ~4 resulted in a lowering of the pseudo-peroxidase activity of metHb both at physiological pH and at meat pH (6.5–6.8). Based on our SDS-PAGE data (Fig. 5D), there was a mild proteolysis of Hb during the first gastric phase, which could have yielded an iron-cycling similar to that described above.

Fish is rich in long chained (LC) n-3 PUFAs, which have been connected to documented beneficial health effects (Zhong, et al., 2020). However, just as lipid oxidation of fish prior to digestion results in a lowered nutritional value, lipid oxidation during digestion will do the same, and in addition give rise to potential toxicological effects due to the reactive aldehydes formed (Tullberg, et al., 2016). There are relatively few human studies focusing on adverse health effects of lipid oxidation-derived aldehydes during digestion. However as we recently reviewed (Tullberg & Undeland, 2021), the cytotoxicity and reactivity of the lipid oxidation markers followed in this study have been reported both in cell- and animal studies. For example, Guéraud et al. (2015) investigated the impact of a "peroxidable" diet on oxidative stress biomarkers in rats by giving them two edible oils (fish oil versus safflower oil) combined with heme iron or ferric citrate. Results showed that heme iron plus fish oil gave 5-fold as high MDA-levels compared to ferric citrate alone in the GI tract of the rats. Also, the subsequent fecal extracts showed high toxicity towards immortalized rat rectum carcinoma CMT93 cells. These effects could be linked to the fact that secondary lipid oxidation products are able to react and form adducts with both DNA and protein, and to act as bioactive agents in various ways depending on their concentration (Guillén & Goicoechea, 2008). Although there is no formal regulation on oxidized foods, GOED has suggested a voluntary recommended maximum peroxide value (PV) and anisidine value (AV), of ≤ 5, and ≤ 20 mEq/kg, respectively in food oils (GOED, 2017). No levels for specific aldehydes are set. Although the levels of HHE, HNE and MDA detected in this study are > 10 times lower than levels reported to be harmful to cells (Pillon & Soulage, 2012), these lipid oxidation products have been found to be bioavailable and might still have systemic effects *in vivo* beyond our current knowledge.

5. Conclusion

When trout and bovine Hb was subjected to the pH-cycle of a GI digestion process, gastric acidification to pH 6 promoted auto-oxidation of Hb and facilitated Hb unfolding. At pH 3, the heme group was lost because of disruption of the heme-globin linkage. Trout Hb was more sensitive to acidic pH compared with bovine Hb. In a full static *in vitro* GI digestion model, both trout and bovine Hb were more extensively hydrolyzed in the gastric phase compared to in the duodenal phase, without any clear species-differences. As shown by *SEC*-analyses, peptides between 1.3 and 18 kDa were found at the end of digestion. The documented hemin release was found to be attributed to the pH change rather than to enzyme hydrolysis, however, it is likely that the latter generated peptide-hemin complexes with high solubility. Both the hemin release and the severe proteolytic degradation seen under gastric conditions showed a close relationship with the formation of MDA, HHE, and HNE, suggesting that they are both likely routes to Hb-mediated lipid oxidation in the digestion tract. The fact that gastric hemin-loss was more pronounced for trout-Hb, and that this Hb generated more HHE than bovine Hb in the gastric phase, may however imply a more pronounced role of this phenomena. For both Hbs, it is suggested that antioxidants are added to the product or meal where they appear, to counteract potential toxic effects from lipid-derived aldehydes.

CRediT authorship contribution statement

Haizhou Wu: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Cecilia Tullberg:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Semhar Ghirmai:** Investigation, Writing – review & editing. **Ingrid Undeland:** Conceptualization, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the Swedish research council for sustainable development (FORMAS) grant nos. 18-7850/1 and 2016-0118.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133356.

References

Abdollahi, M., Axelsson, J., Carlsson, N.-G., Nylund, G. M., Albers, E., & Undeland, I. (2019). Effect of stabilization method and freeze/thaw-aided precipitation on structural and functional properties of proteins recovered from brown seaweed (Saccharina latissima). *Food Hydrocolloids, 96*, 140–150.

Aranda, R., Cai, H., Worley, C. E., Levin, E. J., Li, R., Olson, J. S., … Richards, M. P. (2009). Structural analysis of fish versus mammalian hemoglobins: Effect of the heme pocket environment on autooxidation and hemin loss. *Proteins: Structure, Function, and Bioinformatics, 75*(1), 217–230.

Benesch, R. E., Benesch, R., & Yung, S. (1973). Equations for the spectrophotometric analysis of hemoglobin mixtures. *Analytical Biochemistry, 55*(1), 245–248.

Bertolotti, M., Carfora, V., & Catellani, P. (2020). Different frames to reduce red meat intake: The moderating role of self-efficacy. *Health communication, 35*(4), 475–482.

Buzala, M., Slomka, A., & Janicki, B. (2016). Heme iron in meat as the main source of iron in the human diet. *Journal of Elementology, 21*(1), 303–314.

Carlsen, C. U., & Skibsted, L. H. (2004). Myoglobin species with enhanced prooxidative activity is formed during mild proteolysis by pepsin. *Journal of Agricultural and Food Chemistry, 52*(6), 1675–1681.

Caron, J., Chataigné, G., Gimeno, J. P., Duhal, N., Goossens, J. F., Dhulster, P., … Flahaut, C. (2016). Food peptidomics of in vitro gastrointestinal digestions of partially purified bovine hemoglobin: Low-resolution versus high-resolution LC-MS/MS analyses. *Electrophoresis, 37*(13), 1814–1822.

Cerbone, A., Toaldo, C., Laurora, S., Briatore, F., Pizzimenti, S., Dianzani, M. U., … Barrera, G. (2007). 4-Hydroxynonenal and PPARγ ligands affect proliferation, differentiation, and apoptosis in colon cancer cells. *Free Radical Biology and Medicine, 42*(11), 1661–1670.

Drabkin, D. L., & Austin, J. H. (1935). Spectrophotometric studies V. a technique for the analysis of undiluted blood and concentrated hemoglobin solutions. *Journal of Biological Chemistry, 112*(1), 105–115.

Ghirmai, S., Eriksson, L., Wu, H., Axelsson, M., & Undeland, I. (2020). Improving the stability of red blood cells in rainbow trout (*Oncorhynchus mykiss*) and herring (*Clupea harengus*): Potential solutions for post-mortem fish handling to minimize lipid oxidation. *Food and Bioprocess Technology, 13*(8), 1344–1355.

Glahn, R. P., Wortley, G. M., South, P. K., & Miller, D. D. (2002). Inhibition of iron uptake by phytic acid, tannic acid, and $ZnCl_2$: Studies using an in vitro digestion/Caco-2 cell model. *Journal of Agricultural and Food Chemistry, 50*(2), 390–395.

Guéraud, F., Taché, S., Steghens, J.-P., Milkovic, L., Borovic-Sunjic, S., Zarkovic, N., … Pierre, F. (2015). Dietary polyunsaturated fatty acids and heme iron induce

oxidative stress biomarkers and a cancer promoting environment in the colon of rats. *Free Radical Biology and Medicine, 83*, 192–200.
Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α, β-unsaturated aldehydes and their study in foods: A review. *Critical Reviews in Food Science and Nutrition, 48*(2), 119–136.
Halliwell, B., Zhao, K., & Whiteman, M. (2000). The gastrointestinal tract: A major site of antioxidant action? *Free radical research, 33*(6), 819–830.
Kristinsson, H. G. (2002). Acid-induced unfolding of flounder hemoglobin: Evidence for a molten globular state with enhanced pro-oxidative activity. *Journal of Agricultural and Food Chemistry, 50*(26), 7669–7676.
Kristinsson, H. G., & Hultin, H. O. (2004). The effect of acid and alkali unfolding and subsequent refolding on the pro-oxidative activity of trout hemoglobin. *Journal of Agricultural and Food Chemistry, 52*(17), 5482–5490.
Larsson, K. (2016). *Oxidation of fish lipids during gastrointestinal in vitro digestion*: Chalmers Tekniska Hogskola (Sweden).
Li, Q., Zhao, D., Liu, H., Zhang, M., Jiang, S., Xu, X., … Li, C. (2020). "Rigid" structure is a key determinant for the low digestibility of myoglobin. *Food Chemistry: X, 7*, Article 100094.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.
Maestre, R., Pazos, M., & Medina, I. (2009). Involvement of methemoglobin (MetHb) formation and hemin loss in the pro-oxidant activity of fish hemoglobins. *Journal of Agricultural and Food Chemistry, 57*(15), 7013–7021.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Dupont, D. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & Function, 5*(6), 1113–1124.
Pillon, N. J., & Soulage, C. O. (2012). *Lipid Peroxidation by-Products and the Metabolic Syndrome*. Lipid Peroxidation): InTech.
Richards, M. P. (2010). Heme proteins and oxidation in fresh and processed meats. In *Oxidation in foods and beverages and antioxidant applications* (pp. 76–104). Elsevier.
Richards, M. P., Aranda, R., IV, He, C., & Phillips, G. N., Jr (2009). Effect of pH on structural changes in Perch hemoglobin that can alter redox stability and heme affinity. *Journal of Aquatic Food Product Technology, 18*(4), 416–423.
Richards, M. P., & Hultin, H. O. (2002). Contributions of blood and blood components to lipid oxidation in fish muscle. *Journal of Agricultural and Food Chemistry, 50*(3), 555–564.
Sams, L., Paume, J., Giallo, J., & Carrière, F. (2016). Relevant pH and lipase for in vitro models of gastric digestion. *Food & Function, 7*(1), 30–45.
Sicard, J., Mirade, P.-S., Portanguen, S., Clerjon, S., & Kondjoyan, A. (2018). Simulation of the gastric digestion of proteins of meat bolus using a reaction–diffusion model. *Food & Function, 9*(12), 6455–6469.
Sousa, R., Portmann, R., Dubois, S., Recio, I., & Egger, L. (2020). Protein digestion of different protein sources using the INFOGEST static digestion model. *Food Research International, 130*, Article 108996.
Steppeler, C., Haugen, J.-E., Rødbotten, R., & Kirkhus, B. (2016). Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during in vitro digestion of cooked beef, pork, chicken, and salmon. *Journal of Agricultural and Food Chemistry, 64*(2), 487–496.
Tang, Q., Kalsbeck, W. A., Olson, J. S., & Bocian, D. F. (1998). Disruption of the Heme Iron− Proximal Histidine Bond Requires Unfolding of Deoxymyoglobin. *Biochemistry, 37*(19), 7047–7056.
Tullberg, C., Larsson, K., Carlsson, N.-G., Comi, I., Scheers, N., Vegarud, G., & Undeland, I. (2016). Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: Comparison of human and porcine in vitro digestion models. *Food & Function, 7*(3), 1401–1412.
Tullberg, C., & Undeland, I. (2021). *Oxidative stability during digestion. In Omega-3 Delivery Systems* (pp. 449–479). Elsevier.
Tullberg, C., Vegarud, G., & Undeland, I. (2019). Oxidation of marine oils during in vitro gastrointestinal digestion with human digestive fluids–Role of oil origin, added tocopherols and lipolytic activity. *Food Chemistry, 270*, 527–537.
Undeland, I., Kristinsson, H. G., & Hultin, H. O. (2004). Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. *Journal of Agricultural and Food Chemistry, 52*(14), 4444–4451.
Wu, H., Abdollahi, M., & Undeland, I. (2021). Effect of recovery technique, antioxidant addition and compositional features on lipid oxidation in protein enriched products from cod-salmon and herring backbones. *Food Chemistry, 360*, Article 129973.
Wu, H., Ghirmai, S., & Undeland, I. (2020). Stabilization of herring (*Clupea harengus*) by-products against lipid oxidation by rinsing and incubation with antioxidant solutions. *Food Chemistry, 316*, Article 126337.
Wu, H., Sajib, M., & Undeland, I. (2021). Controlling hemoglobin-mediated lipid oxidation in herring (*Clupea harengus*) co-products via incubation or dipping in a recyclable antioxidant solution. *Food Control, 125*, Article 107963.
Wu, H., Xiao, S., Yin, J., Zhang, J., & Richards, M. P. (2021). Mechanisms involved in the inhibitory effects of free fatty acids on lipid peroxidation in turkey muscle. *Food Chemistry, 342*, Article 128333.
Wu, H., Yin, J., Zhang, J., & Richards, M. P. (2017). Factors affecting lipid oxidation due to pig and Turkey hemolysate. *Journal of Agricultural and Food Chemistry, 65*(36), 8011–8017.
Zhong, V. W., Van Horn, L., Greenland, P., Carnethon, M. R., Ning, H., Wilkins, J. T., … Allen, N. B. (2020). Associations of processed meat, unprocessed red meat, poultry, or fish intake with incident cardiovascular disease and all-cause mortality. *JAMA Internal Medicine, 180*(4), 503–512.