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Changes in the Antioxidative Property of Herring (*Clupea harengus*) Press Juice during a Simulated Gastrointestinal Digestion

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The aqueous fraction (press juice, PJ) from herring muscle was recently shown to inhibit hemoglobin-mediated oxidation of washed fish mince lipids during ice storage. As a first step to evaluate potential *in vivo* antioxidative effects from herring PJ, the aim of this study was to investigate whether herring PJ retains its antioxidative capacity during a simulated gastrointestinal (GI) digestion. Press juice from whole muscle (WMPJ) and light muscle (LMPJ) was mixed with pepsin solution followed by stepwise pH adjustments and additions of pancreatin and bile solutions. Digestive enzymes were removed from samples by ultrafiltration (10 kDa). Before, during, and after digestion, samples were analyzed for their peptide content and for antioxidative properties with the oxygen radical absorbance capacity (ORAC) and the low-density lipoprotein (LDL) oxidation assays. From 0 to 165 min of digestion, the content of <10 kDa peptides in WMPJ and LMPJ samples increased 12- and 7-fold, respectively. Further, both samples got ~12.5 times higher ORAC values and gave rise to ~1.3-fold increased lag phase in Cu^{2+}-induced LDL oxidation. The largest changes in peptide content, ORAC values, and LDL oxidation inhibition occurred between 30 and 75 min of digestion, indicating that these parameters might be interrelated. When comparing analytical data obtained after 165 min of digestion with data obtained from analyses of native nondigested PJs, it was found that the data on peptide content, ORAC, and LDL oxidation from digested PJs were 64–69%, 121–161%, and 112–115%, respectively, of those of nondigested PJs. The study thus showed that enzymatic breakdown of PJ proteins under GI-like conditions increases the peroxyl radical scavenging activity and the potential to inhibit LDL oxidation of herring PJs. These data provide a solid basis for further studies of uptake and *in vivo* activities of herring-derived aqueous antioxidants.

KEYWORDS: Herring; *Clupea harengus*; press juice; lipid oxidation; antioxidant; LDL; ORAC; *in vitro* digestion; gastrointestinal

INTRODUCTION

Oxidative stress is a condition that results due to a shift in the balance between oxidants and antioxidants in favor of oxidants. Oxidative stress promotes damage to important cellular constituents, and this may in turn lead to dysfunction and ultimately to aging, disability, and disease. Many epidemiological studies have shown a strong link between increased fruit and vegetable intake and reduced risk for a number of chronic diseases which are associated with oxidative stress (*1*–*3*). It is speculated that the plant-derived antioxidants are responsible for these effects. Literature on animal-derived antioxidants and their health benefits is very limited, while their role in stabilizing food systems has recently been addressed, especially in the marine area. A few studies have for example shown aqueous fractions (press juices, PJ) of cod, haddock (*4*), and herring (*5*) to have strong antioxidative properties against hemoglobin-mediated oxidation in a washed cod mince model system during ice storage. The antioxidants were found to be both of protein and low molecular weight (LMW) nature (*4*, *6*). Herring PJ also prevented reactive oxygen species (ROS) production in human monocytes (*6*).

The gastrointestinal (GI) tract is known to be a main site of oxidation in the human body because of exposure to food derived iron, copper, H_2O_2, heme, lipid peroxides, nitric oxide, and aldehydes (*7*). The oxidative conditions in the GI tract might influence the properties of food-derived antioxidants. For example, polyphenols from tea showed reduced antioxidative property after an *in vitro* digestion study (*8*). To further explore potential health effects provided by the antioxidants in fish PJ, it thus is essential to have the knowledge of their stability in the GI tract. *In vitro* methods for simulating the human digestive

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tract are being extensively used at present since they are rapid, safe, and do not have the same ethical restrictions as in *in vivo* methods. Such methods have been used for testing angiotensin converting enzyme (ACE) inhibitory properties of egg white hydrolysate (*9*) and iron binding properties and peptide release of different meat sources (*10*). To our knowledge, there are no reported studies on changes in the antioxidative properties of fish during GI digestion. The aim of this study was to evaluate how the antioxidative capacity of a <10 kDa fraction of herring (*Clupea harengus*) whole muscle and light muscle PJ changes during an *in vitro* GI digestion. The antioxidative activity was evaluated using two well-known screening assays, oxygen radical absorbance capacity (ORAC) and the inhibition of the oxidative modification of low-density lipoprotein (LDL) (*11*).

MATERIALS AND METHODS

Chemicals. 3′,6′-Dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one (fluorescein) was obtained from Fluka (Fluka Chemie AG, Buchs, Switzerland). Chloramphenicol, pepsin (P6887), pancreatin (P1750), bile extract (B8631), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), bovine serum albumin, and Folin and Ciocalteu's phenol reagent were obtained from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO). Electrophoresis buffers, standards, and staining and destaining solutions were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). All other reagents used were of analytical grade.

Materials. Ready electrophoresis gels (10–20% Tris-HCl and 16.5% Tris–Tricine) were purchased from Bio-Rad (Bio-Rad Laboratories). Human LDL was purchased from Athens Research and Technology, Athens, GA. Fresh herring (*C. harengus*) fillets, prepared 12 h postmortem, were obtained iced from Paul Mattson AB, Ellös (Sweden). Their post-mortem time when arriving in the laboratory was 30 h.

Preparation of Human LDL. The purchased LDL had been prepared by a sequential flotation ultracentrifugation technique using potassium bromide (KBr) for density adjustment. It was then dissolved in 200 μL of 150 mM NaCl, containing 0.01% EDTA and with pH adjusted to 7.4. The density of LDL was 1.02–1.063 g/mL, and the composition was the following: 78–80% lipid; 22–20% protein; 9.73 mg of cholesterol/mL; 2.37 mg of triglyceride/mL.

Press Juice Preparation. Whole skinless herring fillets and manually removed light muscle were used to obtain whole muscle press juice (WMPJ) and light muscle PJ (LMPJ), respectively. The procedure described by Gunnarsson et al. (*6*) was followed to obtain PJ. In brief, the muscle was minced and then centrifuged at 18300*g* for 2 h at 4 °C. The supernatant obtained was filtered through Munktell's filter paper no. 1003 (Munktell Filter AB, Grycksbo, Sweden) and referred to as PJ. The PJs were stored at −80 °C until use. The LMPJ was dialyzed according to the method described by Gunnarsson et al. (*6*) to obtain a fraction >3.5 kDa which is called high molecular weight PJ (HMW-LMPJ). The LMPJ was ultrafiltered to get a fraction <1 kDa (LMW-LMPJ) using a 250 mL stirred ultrafiltration cell (model 8400; Millipore Corp., Bedford, MA) at 4 °C (*6*). Both HMW-LMPJ and LMW-LMPJ were stored at −80 °C until use.

Analysis of pH, Moisture, Conductivity, and Fat Content. The procedures adapted for measurement of pH, conductivity, and moisture of the different PJ samples are descried elsewhere (*6*). In brief, fat content analyses were done gravimetrically by extracting the PJ with chloroform–methanol (1:2) (*12*).

***In Vitro* Gastrointestinal Digestion Procedure.** A two-stage digestion simulating the human gastric phase and the intestinal phase was carried out by gradient pH adjustment. The *in vitro* digestion trial was performed in 50 mL plastic tubes containing 15 mL of one of the following samples, LMPJ, WMPJ, LMW-LMPJ, or HMW-LMPJ, and 50 mM phosphate buffer with 0.9% NaCl, pH 6.75 (control). To each tube was added 15 mL of a pepsin solution containing 0.462% pepsin (P6887), 49 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 2.4 mM $MgCl_2$, and 3.5 mM K_2HPO_4 (*13*). To prevent bacterial contamination during the digestion process, 7.5 mg of chloramphenicol (250 μg/mL) was also added to each tube.

Table 1. Schedule of the *in Vitro* Digestion Procedure[a]

simulated digestive part	pH	duration, min	addition of enzyme solution: pepsin solution, mL	addition of enzyme solution: pancreatin and bile, mL	sample taken, mL (min)
stomach	5.5	0	15		6 (0)
	5.5	30			8 (30)
	3.8	30			
	2.0	15			6 (75)
small intestine	5.0	30		1.5	6 (105)
	6.5	60			5 (165)

[a] The volumes of the additions were based on an initial meal volume of 15 mL. All samples were centrifuged at 6800*g* for 5 min; supernatants were ultrafiltered at 4000*g* for 15 min using 10 kDa membranes and kept at −80 °C until analysis.

Table 1 shows the schedule used for the *in vitro* digestion trial and for the additional samplings. The pH was adjusted to 5.5 with 3 M HCl, and 6 mL of sample was taken out at 0 min (sample 1). The tubes were incubated in a shaking bath (220 rpm) for 165 min at 37 °C. After 30 min of incubation, 8 mL of sample was taken out (sample 2), and the pH was then adjusted to 3.8 with 3 M HCl. After 60 min, the pH was brought down to 2.0 with 3 M HCl, and after 75 min, 6 mL of sample was taken out (sample 3). Thereafter, 1.5 mL of a pancreatin and bile solution was added that contained 4 g/L pancreatin (P1750), 25 g/L bile extract (B8631), and 0.1 M $NaHCO_3$ (*13*). The pH was then adjusted to 5.0 with 3 M NaOH. In total, the PJ samples were thereby diluted 1.15 times, which was accounted for in the calculations of the results. After 105 min, 6 mL of sample was taken out (sample 4) whereupon the pH was adjusted to 6.5 with 3 M NaOH. After 165 min, the remaining amount in each tube was taken as sample 5. All of the samples (1–5) were immediately put on ice to stop the reaction, and they were then centrifuged at 6800*g* (Multifuge 1S-R; Kendro Laboratory Products, Osterode, Germany) for 5 min to remove large particles. Finally, the supernatants were ultrafiltered (10 kDa MWCO; Vivascience AG, Hannover, Germany) by centrifuging at 4000*g* (Multifuge 1S-R; Kendro Laboratory Products, Osterode, Germany) for 15 min at 4 °C to remove added enzymes (*10*). All filtrates were kept at −80 °C until protein and antioxidant analysis. The digestion trials were repeated three times for each type of PJ. A fourth digestion trial was made only for LMPJ in order to collect some extra samples for electrophoretic analysis. In this trial samples were taken out before centrifugation, right after centrifugation (the supernatant), and after filtering the supernatant with a 10 kDa membrane. All samples were kept at −80 °C until analysis.

Protein Determination. Total protein/peptide content of nondigested and digested PJ samples was determined according to Lowry et al. (*14*) using bovine serum albumin (BSA) as the standard. The measurements were done in triplicate, and the results were expressed as milligrams per milliliter.

Electrophoresis. Samples 1–5 from the digestion of LMPJ, HMW-LMPJ, and LMW-LMPJ, as well as the extra LMPJ samples taken to evaluate the changes during centrifugation and ultrafiltration, were diluted 15 times in 50 mM phosphate buffer, pH 7.4, and prepared for electrophoresis by mixing in 2 volumes of electrophoresis sample buffer. Samples were heated for 5 min at 95 °C and then stored at −80 °C until analysis. The polypeptide profile of all samples was followed with SDS–PAGE according to Laemmli (*15*) in a vertical cell unit at a constant voltage of 200 V. A 16.5% Tris–Tricine/peptide ready gel was used for all samples <10 kDa (LMPJ, HMW-LMPJ, and LMW-LMPJ) and stained with Coomassie Brilliant Blue G-250. For the additional LMPJ samples taken prior to ultrafiltration, a 10–20% Tris-HCl ready gel was used and was stained with Coomassie Brilliant Blue R-250. The gels were stained for 30 min and destained for 2 h with 2–3 times change of destaining solution on a shaking platform to facilitate both staining and destaining. Electrophoratograms were obtained using a calibrated imaging densitometer (Model GS 800; Bio-Rad Laboratories) and analyzed using Bio-Rad Quantity One (version 4.5 software; Bio-Rad Laboratories).

Table 2. pH, Moisture Content, Conductivity, Protein Content, Fat Content, and ORAC Values of Various Fractions of Herring Press Juice[a]

	pH	conductivity (mS/cm)	moisture content (%)	fat content (%)	protein concn (mg/mL)	ORAC value (mmol/L of Trolox equiv)
WMPJ	6.80	15.05	87.6	0.23 ± 0 0.00a	101.0 ± 0.7a	43.0 ± 3.4a
LMPJ	6.76	14.64	87.5	0.20 ± 0.01a	97.4 ± 2.0b	32.3 ± 3.8b
HMW-LMPJ	6.56	17.97	91.8	0.17 ± 0.0b	83.8 ± 0.3c	18.6 ± 3.3c
LMW-LMPJ	6.39	18.53	96.3	0.02 ± 0.02c	1.2 ± 1.3d	3.6 ± 0.2d

[a] Protein and ORAC values were determined in triplicate and expressed as the mean ± SD. Values in the same column followed by different letter are significantly different at $p < 0.05$. Bonferroni post hoc treatment was applied. Key: LMPJ, light muscle PJ; WMPJ, whole muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ.

Oxygen Radical Absorbance Capacity (ORAC). ORAC assay was followed as described by Davalos et al. (*16*). Instead of recording fluorescence every minute for 80 min (*16*) we recorded fluorescence every 30 s for 120 min. Trolox, a synthetic water-soluble derivative of tocopherol, was used as a standard (1–8 μM final concentration). The samples were prediluted 500–1000 times in 75 mM phosphate buffer, pH 7.4. The assay was performed using 96-well, black, FluoroNunc microplates (Nunc, Roskilde, Denmark) on Safire2, a monochromator-based microplate detection system with the software Magellan, both from Tecan Nordic AB (Mölndal, Sweden). The measurements were done in triplicate, and the results are expressed as millimoles per liter of Trolox equivalents.

LDL Oxidation. In this study the LDL oxidation was performed in microplates, and the final volume of assay was 200 μL. The concentrations of LDL and Cu^{2+} had to be chosen carefully so that the maximum of conjugated diene production would come before the samples started to evaporate (*17*). It was also of importance that the maximum level of conjugated dienes was high enough so that differences in absorbance between samples could be observed (*17*). On the basis of preliminary trials, 100 μg of LDL protein/mL and 10 μM $CuSO_4$ were chosen.

A LDL stock solution was prepared by diluting human LDL in 50 mM phosphate buffer containing 0.9% saline (pH 7.4); the protein concentration was 400 μg/mL. Fifty microliters of the LDL solution was added to each 96 well in a transparent UV microplate (CorningInc., Corning, NY) to reach a final concentration of 100 μg of LDL protein/mL. All PJ samples were prediluted 300 times in 50 mM phosphate buffer. Twenty microliters of each sample and blank (50 mM phosphate buffer) was added in triplicate to the plate. Eighty microliters of phosphate buffer was added, and the initial absorbance was measured at 234 nm. To each well was then added 50 μL of 40 μM $CuSO_4$ solution (final concentration 10 μM). The final volume in each well was 200 μL. The plates were incubated at 37 °C for 980 min while the absorbance was measured at 234 nm in 20 min intervals. The initial absorbance value was subtracted from the respective samples. The assay was performed on the same monochromator-based microplate detection system as described above.

The kinetics of LDL oxidation was characterized by three parameters: (i) the length of the lag phase, i.e., the interval (in minutes) between the initial absorbance axis and the intercept of the linear least-squares slope of the curve; (ii) maximum rate of oxidation (V_{max}), i.e., the maximal slope of the absorbance curve in the propagation phase; (iii) maximal accumulation of oxidation products (A_{max}). All of the results of LDL oxidation such as lag phase, V_{max}, and A_{max} are normalized by dividing the respective value with that of the blank in the same plate. The measurements were done in triplicate, and the results are given as the mean ± standard deviation.

Statistical Analysis. The whole *in vitro* digestion procedure was performed three times for all PJs. Within each digestion procedure, three aliquots of the ultrafiltrated digests ($n = 3$) were taken at each sampling point (1–5) and analyzed for proteins, ORAC, and inhibition of LDL oxidation. Experimental values are presented as means ± standard deviations. Differences between the values were evaluated by one-way ANOVA using either Bonferroni or Duncan post hoc multiple comparison treatment. Mean values differing by $p \leq 0.05$ were considered statistically significant. Statistical calculations were done using the software SPSS version 13.

RESULTS AND DISCUSSION

The protocol used for the simulated *in vitro* gastrointestinal digestion had good reproducibility, which was shown by the low relative standard deviation values from replicate digestion determinations.

pH, Moisture, Conductivity, Fat Content, and Protein Content of the Samples Subjected to *in Vitro* GI Digestion. **Table 2** shows data on the pH, conductivity, moisture, fat, protein, and ORAC values for nondigested PJ. The pH ranged from 6.4 in LMW-LMPJ to 6.8 in WMPJ. The conductivity ranged from 14.6 mS/cm for LMPJ to 18.5 mS/cm for LMW-LMPJ. The moisture content was higher in LMW-LMPJ than in the other samples because of removal of higher molecular mass components by ultrafiltration. Further, dilution during the dialysis resulted in higher moisture for dialyzed LMPJ compared to nondialyzed LMPJ. The fat content was around 0.2% in LMPJ, WMPJ, and HMW-LMPJ, but it was only 0.02% in LMW-LMPJ. These differences in fat content among different PJs were significant ($p < 0.05$). The protein concentration was highest in WMPJ (101 mg/mL) followed by LMPJ (97 mg/mL) and HMW-LMPJ (84 mg/mL). The proteins of the PJ are mainly of sarcoplasmic nature (*10*). LMW-LMPJ had the lowest protein concentration (1.2 mg/mL) due to removal of proteins above 1 kDa in ultrafiltration.

Changes in Polypeptide Content during GI Digestion. The protein/peptide content of the <10 kDa fraction of the digested PJ fractions and the control at different times during *in vitro* digestion is presented in **Figure 1**. The initial protein concentrations of the different PJ samples were quite similar (from 2.4 to 4.4 mg/mL) since the >10 kDa fraction, which includes most of the proteins present in the PJs, had been filtered off. After 75 min of digestion, the peptide concentrations of all PJs, except LMW-LMPJ and the control, were significantly increased ($p < 0.05$). No significant change in protein concentration of PJ samples was seen between 75 and 165 min of digestion. **Figure 1** also shows the pH profile used during the *in vitro* digestion trials.

Polypeptide Profile of the Samples during GI Digestion. **Figure 2** shows the electrophoretic pattern of polypeptides in digested samples of LMPJ, HMW-LMPJ, and LMW-LMPJ in a Tris–Tricine/peptide ready gel. Since all of the samples were centrifuged and ultrafiltered with a 10 kDa membrane, the polypeptide bands obtained were below 10 kDa. It can be seen that, in LMPJ 1 (i.e., sample 1, 0 min of digestion), the intensity of polypeptide bands is very low compared to LMPJ 2 (30 min), LMPJ 3 (75 min), LMPJ 4 (105 min), and LMPJ 5 (165 min). The number of peptides formed increased during digestion, and the increase was mainly due to pepsin and pancreatic enzyme activity. This trend seen in terms of poplypeptides was also recorded by Savoie et al. (*18*), who reported a decrease in nitrogen content of a >10 kDa fraction and a simultaneous increase in nitrogen content of a <10 kDa fraction after pepsin and pancreatic digestion of cod proteins. Sorensen et al. (*19*)

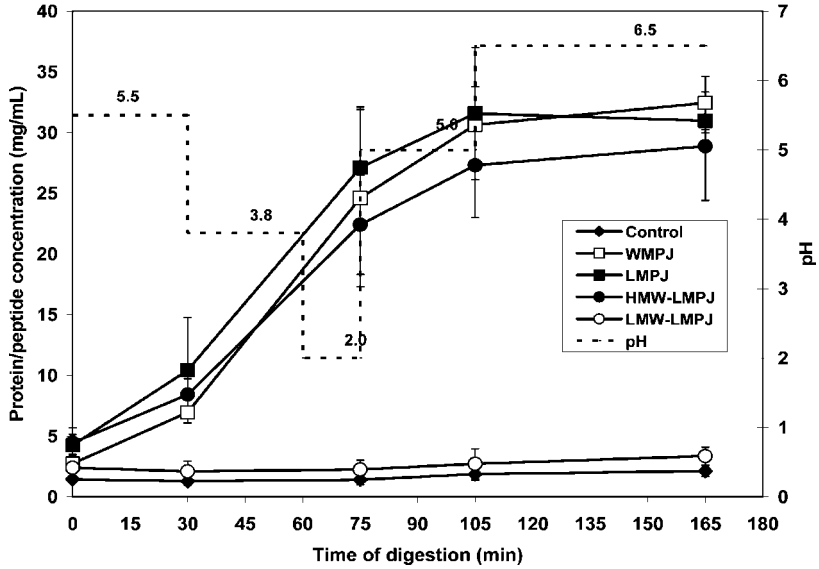

Figure 1. Changes in the protein/peptide concentration (filled lines) of the <10 kDa fractions of the various press juice (PJ) fractions at different times during *in vitro* digestion. Protein analyses were thus done after centrifugation and ultrafiltration of the samples. Key: LMPJ, light muscle PJ; WMPJ, whole muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ. The dotted lines show the pH profile used during the *in vitro* digestion process.

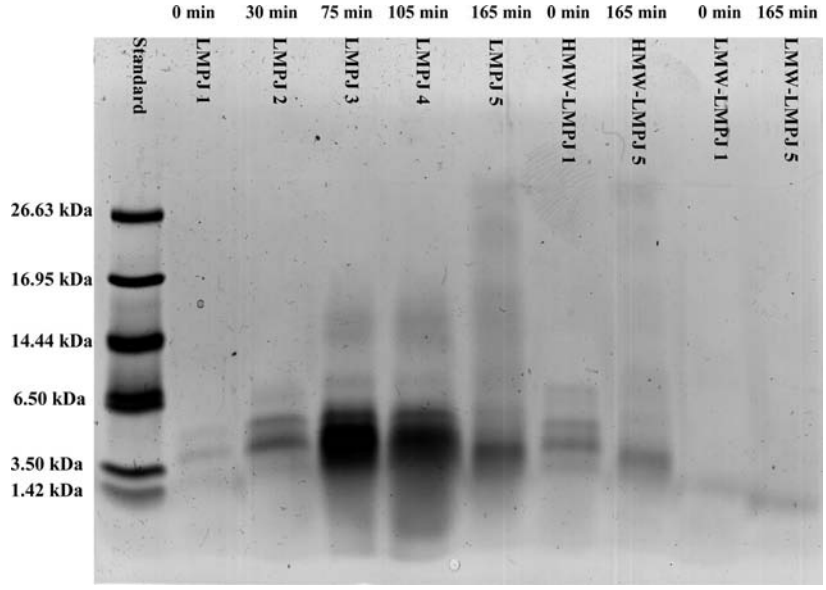

Figure 2. Polypeptide profile of digested LMPJ, HMW-LMPJ, and LMW-LMPJ taken during different times of digestion: (lane Standard) Bio-Rad polypeptide standard; (lane LMPJ 1) <10 kDa fraction of 0 min digested LMPJ; (lane LMPJ 2) <10 kDa fraction of 30 min digested LMPJ; (lane LMPJ 3) <10 kDa fraction of 75 min digested LMPJ; (lane LMPJ 4) <10 kDa fraction of 105 min digested LMPJ; (lane LMPJ 5) <10 kDa fraction of 165 min digested LMPJ; (lane HMW-LMPJ 1) <10 kDa fraction of 0 min digested HMW-LMPJ; (lane HMW-LMPJ 5) <10 kDa fraction of 165 min digested HMW-LMPJ; (lane LMW-LMPJ 1) <10 kDa fraction of 0 min digested LMW-LMPJ, and (lane LMW-LMPJ 5) <10 kDa fraction of 165 min digested LMW-LMPJ. The gel used was 16.5% Tris–Tricine peptide ready gel. Key: LMPJ, light muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ.

reported an increase in peptides of <7 kDa that contributed to more than 90% of the protein after pepsin and pancreatin digestion of heat-treated pork meat. Most polypeptides formed in the later stages of the GI digestion had a molecular mass around 4–5 kDa. The intensity of the bands increased until 75 min of digestion (LMPJ 3) and then decreased. This effect might be due to peptides getting so small after prolonged digestion that they escaped from the gel. The HMW-LMPJ and LMW-LMPJ were only tested after 0 and 165 min of digestion. HMW-LMPJ 1 (0 min) had a few faint bands between 3.5 and 6.5 kDa, and HMW-LMPJ 5 (165 min) had only one band of 4 kDa size. LMW-LMPJ 1 (0 min) and LMW-LMPJ 5 (165 min) each had a faint band of 1 kDa.

Figure 3 gives the relative distribution of proteins/peptides among the various sample fractions taken out at different times during simulated GI digestion of herring LMPJ. Note that at the start of digestion nearly 70% of the initial proteins/peptides of the digested LMPJ ended up in the supernatant, compared to only 3% in the ultrafiltrate. As the time of digestion then increased, the percentage of proteins/peptides increased both in the supernatant and in the ultrafiltrate. This indicates higher solubility and smaller size, respectively. The electrophoretic pattern of polypeptides in LMPJ samples taken at 0, 75, and 165 min during digestion is shown in **Figure 4**. Samples were analyzed before centrifugation and ultrafiltration treatment, after centrifugation, and after ultrafiltration with 10 kDa cutoff

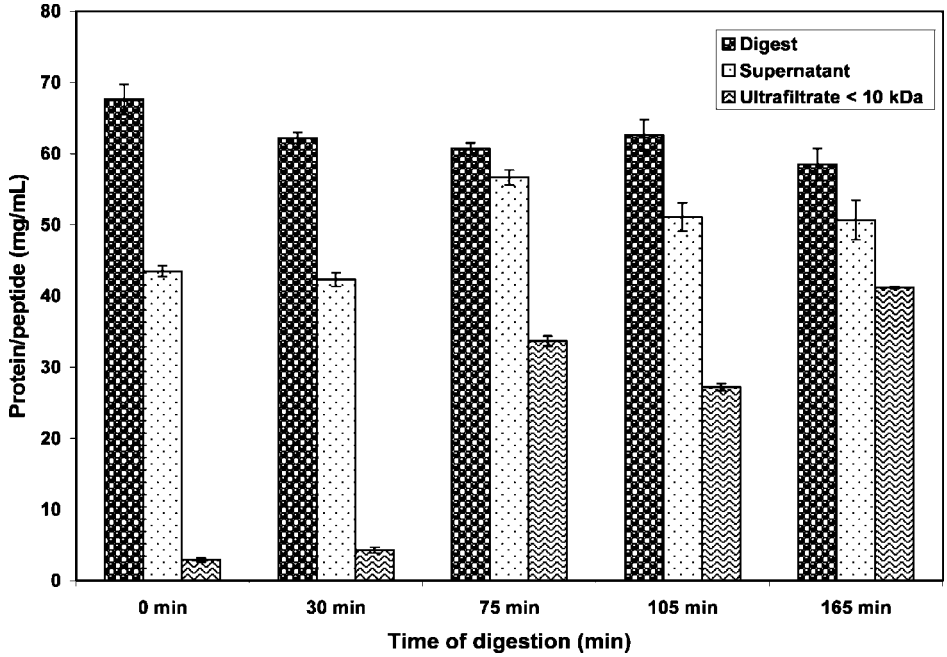

Figure 3. Distribution of protein/peptides among three fractions: digest; supernatant; and ultrafiltrate obtained when samples of digested herring LMPJ were taken at different times of simulated gastrointestinal digestion. Values are the mean of triplicate determination ± SD.

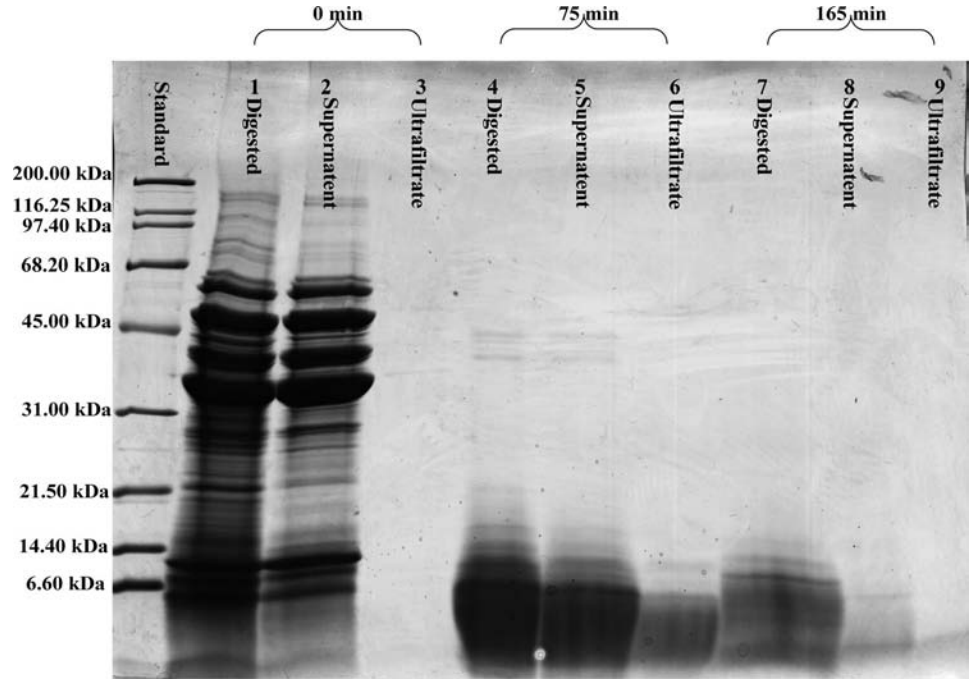

Figure 4. Electrophoretic pattern of digested fractions of LMPJ at different times of digestion: (lane Standard) Bio-Rad broad range molecular mass; (lane 1) 0 min digested LMPJ; (lane 2) the supernatant from centrifugation of 0 min digested LMPJ; (lane 3) the ultrafiltrate from 0 min digested LMPJ (<10 kDa); (lane 4) 75 min digested LMPJ; (lane 5) the supernatant from centrifugation of 75 min digested LMPJ; (lane 6) the ultrafiltrate from 75 min digested LMPJ (<10 kDa); (lane 7) 165 min digested LMPJ; (lane 8) the supernatant from centrifugation of 165 min digested LMPJ; (lane 9) the ultrafiltrate from 165 min digested LMPJ (<10 kDa). The gel used was 10–20% Tris-HCl ready gel.

membrane. The LMPJ sample at the start of digestion had numerous bands ranging from 181 kDa to below 5 kDa size (**Figure 4**, lane 1). This was in accordance with Gunnarsson et al. (*6*), who reported herring PJ to comprise many peptides distributed over a broad range of molecular size. The supernatant resulting from centrifugation of the 0 min digested LMPJ sample also had numerous bands (**Figure 4**, lane 2), showing that most of its proteins/peptides were soluble. That the number and intensity of bands was less compared to the previous lane indicated the removal of some insoluble proteins during centrifugation. The ultrafiltrate of the 0 min digested LMPJ sample had almost no bands (**Figure 4**, lane 3), indicating that all of the proteins were still undigested. After 75 min of digestion, the digest comprised mostly of peptides having a molecular mass <15 kDa (**Figure 4**, lane 4). The corresponding supernatant (**Figure 4**, lane 5) and the ultrafiltrate (**Figure 4**, lane 6) of 75 min digested LMPJ also had numerous peptides <15 kDa, but the ultrafiltrate was significantly less dense compared to the digest (**Figure 4**, lane 4). After 165 min of digestion of LMPJ, the number and the intensity of bands

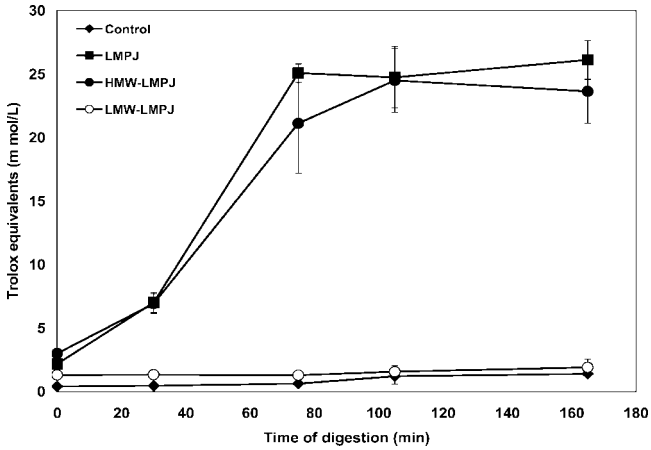

Figure 5. Changes in the ORAC value of the <10 kDa fractions of various press juice (PJ) fractions at pH 7.4 at different times during *in vitro* digestion. Key: LMPJ, light muscle PJ; WMPJ, whole muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ.

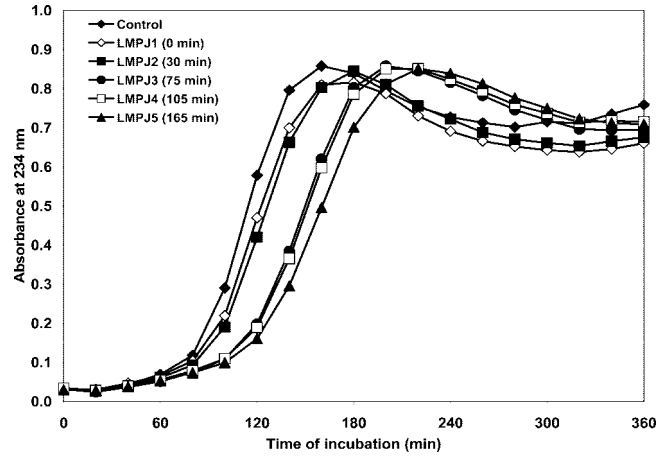

Figure 6. Representative absorbance curve obtained during Cu^{2+}-induced LDL oxidation. The concentration of LDL and Cu^{2+} was 100 μg of LDL protein/mL and 10 μM $CuSO_4$, respectively. Samples shown are the control (50 mM phosphate buffer of pH 7.4) and samples fortified with <10 kDa fractions of LMPJ samples taken at different times during one digestion trial (**Table 1**). All samples were diluted 300 times in the final analysis.

decreased in the digest, the supernatant, and ultrafiltrate (**Figure 4**, lanes 7–9). From **Figures 1**–**4**, it was clear that the amount of peptides ending up in the supernatant and then in the ultrafiltrate was increased over 165 min of digestion; however, the smaller peptides may have passed through the electrophoresis gel.

Screening for Antioxidative Properties. Both ORAC and LDL oxidation assays were performed at physiological conditions (i.e., 37 °C, pH 7.4). However, the LDL oxidation assay can be regarded physiologically more relevant than ORAC since it involves an oxidation substrate (LDL) which is present in the human body. An advantage with the ORAC and LDL assays is that in both of them the reaction is allowed to go to completion. This makes the results more accurate than in other antioxidative methods that often take a measure only after a certain time. The results from the LDL oxidation assay give information about the different mode of antioxidant action. The ORAC assay, on the other hand, gives an absolute value of the peroxy radical scavenging capacity of a sample. In this work, both antioxidant methods used gave about the same relative order of results between the samples.

Oxygen Radical Absorbance Capacity (ORAC) Tests of the Samples. The ORAC values of the nondigested PJ fractions are presented in **Table 2**. The samples were ranked in the following order: WMPJ > LMPJ > HMW-LMPJ > LMW-LMPJ. Gunnarsson et al. (*6*) have reported similar ORAC values for herring LMPJ, LMW-LMPJ, and HMW-LMPJ fractions. The trend in the response from the ORAC values seemed to follow the protein concentration of the respective samples (**Table 2**). Proteins give high response to the ORAC method when muscle extracts are analyzed (*6*, *20*). This is most likely due to the ability of the protein/peptide thiol groups to donate a hydrogen atom to free radicals (*21*). The ORAC values of the <10 kDa fraction of the different digested fractions of PJ and the control at different times during *in vitro* digestion are presented in **Figure 5**. LMPJ samples taken at 0, 30, and 75 min of digestion were significantly different ($p < 0.05$) from each other. The ORAC values increased up to 75 min but then stayed fairly stable. The ORAC values had increased 10.9-, 12.0-, and 6.9-fold after 165 min of digestion of LMPJ, WMPJ, and HMW-LMPJ, respectively. However, in LMW-LMPJ and the control, no changes in ORAC were seen. The ORAC results are not illustrated for WMPJ samples since LMPJ and WMPJ samples taken at different times of digestion showed similar values (**Figure 5**). The fact that the increase in the ORAC values for LMPJ, WMPJ, and HMW-LMPJ followed a similar kinetics as the increase in peptide content (**Figure 1**) indicates that the <10 kDa peptides formed during digestion can scavenge peroxy radicals. However, while the ORAC value of the LMPJ had increased 10.9-fold after 165 min, the protein/peptide content of this sample had increased only 6.4-fold. This indicates that it is not just the peptide quantity that causes the ORAC value increase but also the increase in the amount of peptides that might act as peroxy radical scavengers (*22*).

Peptides and amino acids are known to have significant antioxidant properties, and generally they function as synergists or primary antioxidants (*23*). Examples of antioxidative amino acids are histidine, tyrosine, methionine, and cystine (*24*, *25*). Especially histidine is known to exhibit strong radical scavenging activity which could be attributed to its imidazole ring's hydrogen-donating tendency (*26*). The antioxidant activity of most amino acids depends on the pH (*25*). Here, the samples were buffered to pH 7.4 before analysis, so there is no difference in pH between the samples at different times of digestion that can contribute to the differences seen in the ORAC values. Examples of well-studied antioxidative peptides are the dipeptides carnosine (*27*) and anserine (*28*), as well as the tripeptide glutathione. The amounts of some of these kinds of peptides are likely to increase during digestion of sarcoplasmic herring proteins. The general digestion of the PJ proteins into peptides might also expose previously hidden amino acid residues and side chains like thiol groups with antioxidative properties. The fact that the increase in ORAC values is nonsignificant in the later stages of the digestion indicates that the proteins already have been digested to a degree where all of the antioxidative amino acid residues or side chains have been exposed.

LDL Oxidation. A typical LDL oxidation curve for a control and samples fortified with the <10 kDa fraction of LMPJ samples taken at different times of digestion is presented in **Figure 6**. The kinetics seen were similar to those previously described for Cu^{2+}-induced LDL oxidation (*29*–*32*). In brief, three consecutive phases were seen: a lag phase during which diene absorption increased slowly, a propagation phase with a rapid increase in diene absorption, and a diene decomposition phase. **Figure 6** shows that the control and the LMPJ samples

Table 3. Normalized Lag Phase, V_{max}, and A_{max} of Cu^{2+}-Induced LDL Oxidation in the Presence of Various Nondigested Press Juice (PJ) Fractions at pH 7.4[a]

	lag/lag$_{blank}$	$V_{max}/V_{max,blank}$	$A_{max}/A_{max,blank}$
LMPJ	1.17 ± 0.04a	1.02 ± 0.11a	1.05± 0.02a
WMPJ	1.19 ± 0.04a	0.99 ± 0.05a	1.04± 0.06a
HMW-LMPJ	1.13 ± 0.03ab	1.03 ± 0.08a	1.01± 0.05a
LMW-LMPJ	1.04 ± 0.04b	0.99 ± 0.08a	1.00± 0.04a

[a] All of these PJs were prediluted 600 times for the assay. Values were determined in triplicate and expressed as the mean ± SD. Values in the same column followed by different letter are significantly different at $p < 0.05$. Duncan post hoc treatment was applied. Key: LMPJ, light muscle PJ; WMPJ, whole muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ.

digested for 0 and 30 min had the shortest lag phase, while the 165 min digested LMPJ sample had the longest lag phase. It was noted that the absorption after the propagation phase decreased and then increased again. This could be due to the fact that the aldehydes formed during diene decomposition also absorb in the same region as the conjugated dienes. Even though the same LDL batch and the same Cu^{2+} concentrations were used throughout this study, a difference in the lag phase was seen when the LDL assay was repeated (data not shown). This could be due to small variations in room temperature (*17*) and plate preparation time. Furthermore, the LDL purchased was stored in small quantities in separate tubes to use in different experiments. Thus the difference in LDL storage time might also contribute to the aforementioned variations. However, the relative order of inhibition given by the different samples was nearly the same in each trial, strengthening the information obtained about the capacity of digested PJ to slow LDL oxidation.

The lag phase, V_{max}, and A_{max} obtained by the different fractions of nondigested PJ in the LDL oxidation assay are presented in **Table 3**. Lag phases and V_{max} given by the different PJ samples at different times of digestion are shown in panels a and b of **Figure 7**, respectively. Results are not illustrated for WMPJ samples since LMPJ and WMPJ samples taken at different times of digestion showed the same trend in all LDL oxidation parameters. The LMPJ and HMW-LMPJ samples taken after 75 min digestion significantly ($p < 0.05$) extended the lag phase of LDL oxidation and lowered V_{max} compared to the respective samples taken after 30 min of digestion (**Figure 7a**). The LMW-LMPJ and control samples had no significant effect on the LDL oxidation lag phase or V_{max} at any time of digestion. The latter was in contrast to our studies on herring PJ as a food antioxidant. Here LMW components of herring LMPJ were highly antioxidative against hemoglobin-mediated oxidation of washed cod mince under ice storage (*5*). The results indicate that the capacity of the <10 kDa fractions of LMPJ and HMW-LMPJ to prevent LDL diene formation increases in the digestion process. There was no significant change in A_{max} given by any PJs throughout the digestion. It has previously been shown that thiols like Cys-SH and glutathione inhibit Cu^{2+}-mediated LDL oxidation (*33, 34*). Some oxidized and derivatized thiols like diglutathione (GSSG) and methionine have also shown inhibitory effects on LDL oxidation, indicating that thiol compounds need not be reduced to inhibit LDL oxidation (*35*). Copper-mediated oxidation of LDL can also be inhibited by non-thiol-containing amino acids like lysine, glycine, alanine, serine, and histidine (*34*). Decker et al. (*27*) have shown that peptides such as carnosine can chelate Cu^{2+} and, thus, could contribute to inhibition of LDL oxidation. The histidine residue of carnosine could attribute to the copper chelating effect (*36*).

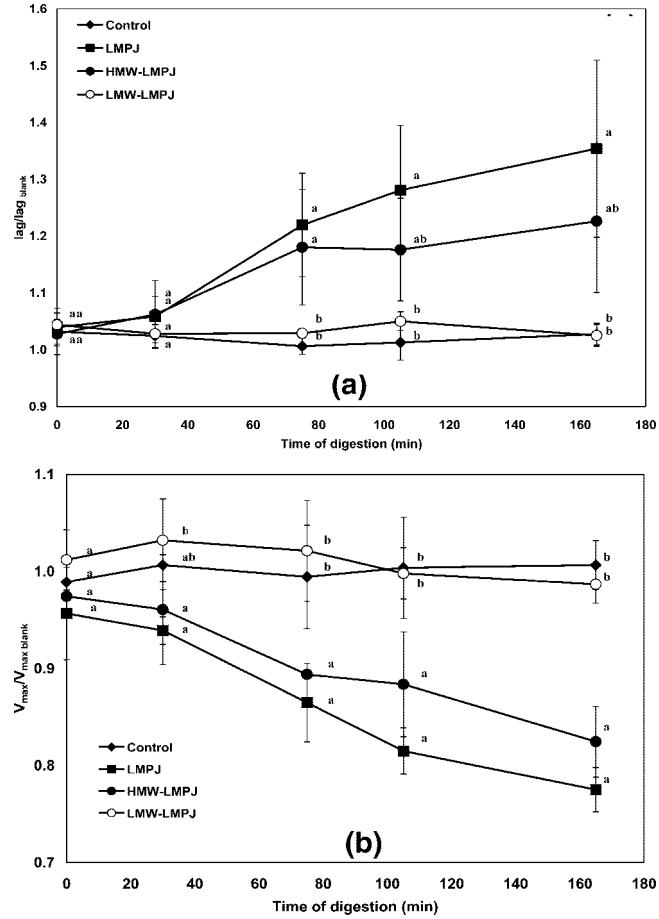

Figure 7. Normalized lag phase (a) and V_{max} (b) of the Cu^{2+}-induced LDL oxidation fortified with the <10 kDa fraction of LMPJ and the HMW and LMW fractions of this PJ at pH 7.4 at different times during *in vitro* digestion. The concentration of LDL and Cu^{2+} was 100 μg of LDL protein/mL and 10 μM $CuSO_4$, respectively. Key: LMPJ, light muscle PJ; WMPJ, whole muscle PJ; HMW-LMPJ, high molecular weight fraction of LMPJ; LMW-LMPJ, low molecular weight fraction of LMPJ. Points at a given time followed by different letters are significantly different from each other at $p < 0.05$. Duncan post hoc treatment was applied.

Saiga et al. (*37*) reported a Cu^{2+} chelating property of meat hydrolysates. These hydrolysates carried acidic and/or basic amino acids that were ascribed an important role in metal chelating. Je et al. (*22*) reported some peptides of 672 Da mass derived from hydrolysis of Alaska pollack frame protein that showed radical scavenging property. Studies on the capacity of herring PJ to scavenge reactive oxygen species production by human monocytes followed the order native LMPJ > HMW-LMPJ > LMW-LMPJ (*6*), thus indicating low antioxidaitve activity of LMW-LMPJ. The effect seen from PJ samples on LDL oxidation could thus be a result of several different antioxidant mechanisms, i.e., both metal chelating and radical scavenging provided, e.g., by thiols, free amino acids, and peptides. Pinchuk and Lichtenberg (*31*) have explained the mode of action of antioxidatants in the LDL oxidation assay. In brief, a prolonged lag phase, without any effect on V_{max} and A_{max}, indicates that the mechanism of the antioxidant involves the scavenging of free radicals or reduction in transfer of reactive intermediates. If the lag phase is prolonged and V_{max} is reduced by an antioxidant, without modifying A_{max}, the mechanism involves either the binding of Cu^{2+} ions or the blocking of LDL Cu^{2+} binding sites. If there is a reduction of the A_{max}, it is indicated that an antioxidant catalyzes nonradical decomposition of hydroperoxide. As the PJ samples gave a prolonged LDL

oxidation lag phase and a decreased V_{max}, without modifying A_{max}, the mode of action in the LDL oxidation assay could be either the binding of Cu^{2+} ions or the blocking of LDL Cu^{2+} binding sites on the LDL.

Comparison between Digested and Nondigested Samples. When comparing analytical data obtained after 165 min digestion with data obtained from nondigested PJs, it was found that the data on peptide content, ORAC, and LDL oxidation given by digested PJs were 64–69%, 121–161%, and 112–115%, respectively, of those of nondigested PJs.

This study thus showed that enzymatic breakdown of PJ proteins under GI-like conditions increases their peroxy radical scavenging activity and the ability to prevent Cu^{2+}-induced LDL oxidation. Altogether, the data provide a solid basis for further studies of uptake and *in vivo* activities of herring-derived aqueous antioxidants.

ABBREVIATIONS USED

PJ, press juice; ROS, reactive oxygen species; ORAC, oxygen radical absorbance capacity; LDL, low-density lipoprotein; LMW, low molecular weight; WMPJ, whole muscle PJ; LMPJ, light muscle PJ; HMW-LMPJ, high molecular weight fraction from LMPJ; LMW-LMPJ, low molecular weight fraction from LMPJ; A_{max}, absorbance maximum; V_{max}, slope maximum; GSSG, diglutathione.

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