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Bioactivity of Cod and Chicken Protein Hydrolysates before and after *in vitro* Gastrointestinal Digestion

Polona Jamnik^{1*}, Katja Istenič¹, Tatjana Koštomaj¹, Tune Wulff², Margrét Geirsdóttir³, Annette Almgren⁴, Rósa Jónsdóttir³, Hordur G. Kristinsson^{3,5} and Ingrid Undeland⁴

¹Biotechnical Faculty, Department of Food Science and Technology, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

²National Food Institute, Technical University of Denmark, Anker Engelunds Vej 1, DK-2800 Kgs. Lyngby, Denmark

³Matis Ltd, Icelandic Food and Biotech R&D, Vinlandsleid 12, IS-113 Reykjavík, Iceland

⁴Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-41296 Göteborg, Sweden

⁵Department of Food Science and Human Nutrition, University of Florida, 572 Newell Drive, Gainesville, FL 32611, USA

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Summary

Bioactivity of cod (*Gadus morhua*) and chicken (*Gallus domesticus*) protein hydrolysates before and after *in vitro* gastrointestinal (GI) digestion was investigated using yeast *Saccharomyces cerevisiae* as a model organism. Both hydrolysates were exposed to *in vitro* GI digestion prior to cellular exposure to simulate digestion conditions in the human body and therefore investigate the role of modulations in the GI tract on the cell response. The effect of digested and undigested hydrolysates on intracellular oxidation, cellular metabolic energy and proteome level was investigated. No difference in the effect on intracellular oxidation activity was obtained between cod and chicken hydrolysates, while higher affect on intracellular oxidation was provided by digested hydrolysates, with relative values of intracellular oxidation of cod of (70.2±0.8) and chicken of (74.5±1.4) % than by undigested ones, where values of cod and chicken were (95.5±1.2) and (90.5±0.7) %, respectively. Neither species nor digestion had any effect on cellular metabolic energy. At proteome level, digested hydrolysates gave again significantly stronger responses than undigested counterparts; cod peptides here also gave somewhat stronger response than chicken peptides. The knowledge of the action of food protein hydrolysates and their digests within live cells, also at proteome level, is important for further validation of their activity in higher eukaryotes to develop new functional food ingredients, such as in this case chicken and cod muscle-derived peptides.

Key words: protein hydrolysates, cod, chicken, *in vitro* gastrointestinal digestion, yeast, proteomics

*Corresponding author: Phone: +386 1 320 3729; Fax: +386 1 256 5782; E-mail: polona.jamnik@bf.uni-lj.si

ORCID IDs: 0000-0001-7648-4767 (Jamnik), 0000-0003-2133-6737 (Istenič), 0000-0003-4922-2811 (Koštomaj), 0000-0002-8822-1048 (Wulff), 0000-0002-8760-8697 (Geirsdóttir), 0000-0002-1663-4855 (Almgren), 0000-0002-5965-4469 (Jónsdóttir), 0000-0002-3277-3433 (Kristinsson), 0000-0002-9732-3644 (Undeland)

Introduction

Protein hydrolysates containing bioactive peptides show potential use as functional food ingredients for health promotion and disease risk reduction. They occur naturally, *e.g.* in dairy and muscle food sources, and are also released during gastrointestinal (GI) digestion or food processing (1,2). Both chicken- and fish-derived peptides had been shown to have antioxidant, antihypertensive, antithrombotic, anticoagulant and immunomodulatory effects (3); therefore, they have high potential as a source of bioactive ingredients.

In spite of recent findings suggesting bioactivity of fish-, chicken- and other muscle-derived peptides, detailed studies at the cellular and specifically at molecular level, which enable better insight into the action in the cell, are still quite sparse. To the best of our knowledge there are no published studies linking muscle-derived peptides to the proteomic response in cells. Besides proteomic response, this study investigates the effects of cod and chicken protein hydrolysates on intracellular oxidation (*i.e.* antioxidant activity) and cellular metabolic energy using yeast *Saccharomyces cerevisiae* in the stationary phase as a model organism, where yeast cells resemble the cells of multicellular organisms in important aspects *e.g.* most energy comes from mitochondrial respiration, it goes into G₀ phase, and oxidative damage accumulates over time (4). Furthermore, both hydrolysates were exposed to *in vitro* GI digestion prior to cellular exposure to simulate the digestion conditions in the human body and therefore investigate the role of enzymatic breakdown and other modulations in the GI tract on the responses given by the peptides. Namely, such simulated digestion studies of treatment of cells with digests of both cod and chicken have not been reported earlier.

Materials and Methods

Chemicals and reagents

Immobilized pH gradient (IPG) buffer and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from GE Healthcare (Little Chalfont, UK). Phosphate-buffered saline (PBS) was from Oxoid (Altrincham, UK). Sodium dodecyl sulphate (SDS), glycerol, thiourea, urea, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), dithiothreitol (DTT), iodoacetamide (IAA) and Bromophenol Blue were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of cod and chicken protein isolates and hydrolysates, and determination of their amino acid composition

Fresh cod (*Gadus morhua*) filets were obtained from Noatun, a grocery store in Reykjavik, Iceland, and fresh chicken (*Gallus domesticus*) breasts from the producer Feriskir kjuklingar (Reykjavik) were purchased from Hagkaup, a grocery store in Reykjavik.

Protein isolates of cod and chicken were prepared by alkali-aided pH-shift processing according to Kristinsson *et al.* (5) with some modifications described by Jónsdóttir *et al.* (6). Briefly, cod fillet/chicken breast was homogenized in water and the pH of the homogenate was adjust-

ed to 11. Insoluble material was removed, while the soluble proteins were precipitated by adjusting pH of the filtrate to 5.5. Cod and chicken protein hydrolysates were produced by Protamex (Novozymes, Bagsvaerk, Denmark) in the enzyme (E)/substrate (S) ratio 1:50. Hydrolysis was performed at 45 °C and pH=8.1 for 6 h. Then the enzyme was inactivated at 95 °C for 10 min. After putting the sample on ice, centrifugation was carried out (30 min, 10 000×g, Avanti J-20 XPI centrifuge; Beckman Coulter, Indianapolis, IN, USA) to collect the soluble fraction and then the pH was adjusted to 7.2. The sample was freeze-dried (Genesis Pilot Lyophilizer, SP Scientific, Warminster, PA, USA) and kept at –80 °C until further analysis.

The amino acid composition of both protein hydrolysates was determined by an external, accredited lab (Eurofins Scientific, Luxembourg, Luxembourg) using standardized methodology, ISO 13903:2005 (7). In short, samples were hydrolyzed in aqueous hydrochloric acid (Sigma-Aldrich) or oxidized with hydrogen peroxide and formic acid (Sigma-Aldrich) at cold temperature. In both cases, amino acids were then separated in an amino acid analyzer Pinnacle PCX derivatization instrument (Pickering Laboratories, Inc., Mountain View, CA, USA) attached to an ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) and detection was carried out at 440 and 570 nm following post-column derivatization with ninhydrin reagent (Sigma-Aldrich). The results are expressed in g of amino acids per 100 g of dry mass.

In vitro gastrointestinal digestion of cod and chicken protein hydrolysates

Cod and chicken protein hydrolysates were digested according to the two-step static *in vitro* GI digestion method described by Tibäck *et al.* (8). In order to avoid the risk of bile salts in the digests affecting the yeast cells with which the digests were to be incubated, samples were digested with only 25 % of the bile extract that was described in the paper by Tibäck *et al.* (8). Blank digestions consisted of digestive juice (electrolyte, digestive enzymes and bile acids) instead of hydrolysate, and were treated exactly as the hydrolysate samples. Final digests were kept at –80 °C until exposure studies in yeast cells.

Yeast strain, cultivation and treatment

The yeast *S. cerevisiae* from Culture Collection of Industrial Microorganisms (Biotechnical Faculty, Ljubljana, Slovenia) was used. The yeasts were cultivated in YEPD broth (Sigma-Aldrich) at 28 °C and 220 rpm on a rotary shaker (Multitron, Bottmingen, Switzerland) until the stationary phase, when the cells were suspended in PBS at a concentration of 10⁸ cells/mL (9). Yeast cells were exposed to cod and chicken protein hydrolysate (before and after digestion) in concentration of 1.0 mg/mL (in yeast suspension) and corresponding controls (control before digestion was water, and control after digestion was digestive juice).

After 2-hour incubation the effect of both hydrolysates (before and after digestion) was studied at the cellular level by measuring cellular metabolic energy, intracellular oxidation and at proteome level by analyzing mitochondrial proteins.

Determination of intracellular oxidation in yeast cells

Intracellular oxidation was determined by the method of Jakubowski and Bartosz (10) with some modifications described by Cigut *et al.* (9). Briefly, yeast cells from 2-mL cell suspensions were centrifuged (14 000×g, 5 min, 5415 C Centrifuge; Eppendorf, Hamburg, Germany) and washed three times with 50 mM potassium phosphate buffer (pH=7.8). The dye 2',7'-dichlorodihydrofluorescein diacetate was added to the yeast cell suspension prepared in potassium phosphate buffer to reach the final concentration of 10 μM and the cells were incubated for 20 min at 28 °C and 220 rpm. Then the fluorescence (excitation and emission wavelengths were 488 and 520 nm, respectively) was measured using microplate reader Safire II (Tecan, Männedorf, Switzerland). Results are expressed as fluorescence of 80-minute measurement in kinetic mode compared to the corresponding control (yeast cells of undigested samples were treated with water and of digested samples with digestive juice).

Determination of cellular metabolic energy

Cellular metabolic energy, expressed as adenosine triphosphate (ATP) level, was determined by BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 100 μL of BacTiter-Glo™ reagent were added to 100 μL of cell suspension with a concentration of 10⁷ cells/mL and after 5-minute incubation luminescence was measured using the microplate reader Safire II (Tecan). Results are expressed as luminescence of the samples compared to the corresponding control (yeast cells of undigested samples were treated with water and of digested samples with digestive juice).

Analysis of mitochondrial proteome

Yeast cells from 20 mL of cell suspension were centrifuged at 4000×g (322A centrifuge; Domel, Železniki, Slovenia) for 3 min and washed once with PBS. Cytosol/Mitochondria Fractionation Kit (Calbiochem, Merck, Darmstadt, Germany) was used to extract mitochondrial proteins according to manufacturer's instructions with minor modifications. Briefly, zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) were used to break the yeast cells in 1250-μL of 1× cytosol extraction buffer mix by vortexing five times for 1 min with 1-minute intervals on ice. After centrifugation at 800×g and 4 °C for 20 min, the supernatant was transferred to a clean microcentrifuge tube and centrifuged at 10 000×g and 4 °C for 30 min to obtain cytosolic fraction (supernatant). The pellet was washed once with PBS and then resuspended in 50 μL of mitochondria extraction buffer mix to obtain the mitochondrial fraction, which was used to analyze mitochondrial proteins.

After extraction of proteins, 2-D electrophoresis was performed according to Görg (11) with minor modifications described by Cigut *et al.* (9). In brief, the samples were mixed with rehydration solution (7 M urea, 2 M thiourea, 2 % (by mass per volume) CHAPS, 2 % (by volume) immobilised pH gradient (IPG) buffer (pH=4–7), 18 mM dithiothreitol and a trace of Bromophenol Blue) and put on 13-cm IPG strips at pH=4–7 (GE Healthcare). Isoelectric focusing was performed using Multiphore II sys-

tem (GE Healthcare) and then the IPG strips were equilibrated in equilibration buffer (75 mM Tris-HCl, pH=8.8, 6 M urea, 30 % (by volume) glycerol, 2 % (by mass per volume) SDS and a trace of Bromophenol Blue), containing 1 % (by mass per volume) dithiothreitol (15 min), followed by the addition of 4.8 % (by mass per volume) iodoacetamide (15 min). SDS polyacrylamide gel electrophoresis was performed with 12 % running gels using a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments, Holliston, MA, USA).

After staining the gels with SYPRO Ruby (Carlsbad, CA, USA), they were documented using a CAM-GX-CHEMI HR system (Syngene, Cambridge, UK).

2-D Dymension software, v. 2.02 (Syngene) was used for the gel image analysis where the spots were quantified based on their normalized volumes and compared among different samples to give differentially expressed proteins (9), which were then identified using an Ultraflex II mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany). Identifications were based on MS/MS spectra of samples using MASCOT to search the NCBI nr database for similarity with *S. cerevisiae* as described by Larsson *et al.* (12). Briefly, trypsin was used as enzyme, and carboxyamidomethylation of cysteine and oxidation of methionine were used as fixed and partial modifications, respectively. Fragment ion mass tolerance was 0.5 Da and a precursor mass accuracy was 50 ppm.

Statistical analysis

The experiments (intracellular oxidation and cellular metabolic energy) were performed in triplicates. Data are presented as mean relative values±standard deviation. Duncan's multiple range test was used to determine the significant differences ($p \leq 0.05$) among the mean relative values. For proteome analysis, two 2-D gels of each sample were run under the same conditions. Expression changes (fold changes) were considered as significant when the intensity of the corresponding spots differed by more than 1.5-fold in a normalized volume from the corresponding control (yeast cells of undigested samples were treated with water and of digested samples with digestive juice) and statistically significant (Student's *t*-test) at $p \leq 0.05$. Comparison of fold changes of particular proteins between digested and undigested samples (cod and chicken) was done using Student's *t*-test ($p \leq 0.05$).

Results and Discussion

Antioxidant activity in the cells and cellular metabolic energy

Many studies have demonstrated earlier that protein hydrolysates from fish proteins are known for their antioxidant activity (6,13–17). There are fewer reports about antioxidant activity of chicken protein-derived peptides (18,19), and only one study has compared fish with avian muscle hydrolysates (20); however, this study is without an *in vitro* digestion step.

In most of the mentioned studies, antioxidant activity has been determined *in vitro* by measuring DPPH radical scavenging capacity, reducing power assay, metal-chelat-

ing activity assay or by the ability of hydrolysates to inhibit or delay lipid peroxidation in emulsions or muscle minces, which can give valuable information when to use peptides as food stabilizing agents. Regarding antioxidant activity at a cellular level, compounds showing antioxidant activity *in vitro* or in foods do not necessarily possess the same activity in the cells (21). If not injected, there will always be a GI digestion step, where peptides will be modified, *e.g. via* further proteolysis. Therefore, in this study, antioxidant activity of cod and chicken protein hydrolysates before and after *in vitro* GI digestion was determined on a cellular level by measuring intracellular oxidation in the yeast *S. cerevisiae* exposed to digested and undigested samples. Before digestion, both hydrolysates showed a slight decrease in intracellular oxidation compared to control: (95.5±1.2) % (cod) and (90.5±0.7) % (chicken), which was more pronounced after digestion, in both cod and chicken samples, (70.2±0.8) and (74.5±1.4) %, respectively. As before, even after digestion, no differences between both hydrolysates were observed (Fig. 1). In the study of Centenaro *et al.* (20), fish (*Umbrina canosai*) hydrolysates generally showed higher antioxidant activity in *in vitro* tests (*e.g.* ABTS and DPPH radical scavenging) and in a meat system than chicken hydrolysates, which was ascribed to more sulphur-containing amino acids and hydrophobic amino acids in the fish hydrolysate. Here both protein hydrolysates showed similar amino acid profiles (Table 1) except for the content of histidine, serine and methionine, which differed by ≥20 % between samples; cod protein hydrolysate had 20 % lower content of histidine than chicken protein hydrolysate, while the content of serine and methionine was higher in cod than in chicken protein hydrolysate (20 and 23 %, respectively). All these three amino acids are known to have antioxidant properties (22–24). Although the content of two amino acids, methionine and serine, was higher in cod than in chicken protein hydrolysate, their antioxidant activity was comparable, and it was more pronounced after digestion. This indicates that by modification or further hy-

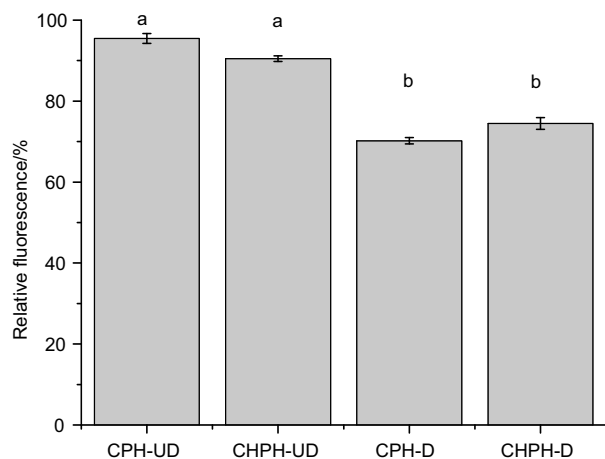


Fig. 1. Intracellular oxidation of yeast *Saccharomyces cerevisiae* exposed to undigested and digested cod (CPH-UD and CPH-D, respectively) and chicken protein hydrolysates (CHPH-UD and CHPH-D, respectively). Results are expressed as average values of relative fluorescence±standard deviation (S.D.), $N=3$. Values followed by the different letter are statistically significantly different ($p \leq 0.05$), as measured by Duncan's test

Table 1. Amino acid composition on dry mass basis of cod and chicken protein hydrolysates. The third column shows the ratio between cod protein hydrolysate (CPH) and chicken protein hydrolysate (CHPH) for each amino acid or groups of amino acids

Amino acid	$w/(g/100\text{ g})^*$		CPH/CHPH
	CPH	CHPH	
Alanine	5.12	4.83	1.06
Arginine	5.44	5.19	1.05
Asparagine	9.70	8.45	1.15
Glutamine	14.70	12.90	1.14
Glycine	3.30	3.08	1.07
Histidine	1.92	2.39	0.80
Isoleucine	3.89	4.08	0.95
Leucine	7.35	6.96	1.06
Lysine	8.98	8.07	1.11
Phenylalanine	3.25	3.45	0.94
Proline	2.77	2.71	1.02
Serine	3.95	3.29	1.20
Threonine	4.09	3.89	1.05
Tyrosine	3.21	2.87	1.12
Valine	4.40	4.27	1.03
Cysteine	0.88	0.88	1.00
Methionine	2.52	2.05	1.23
HAA	33.39	32.10	1.04
PCAA	16.34	15.65	1.04
NCAA	32.44	28.53	1.14
AAA	6.46	6.32	1.02
SAA	3.40	2.93	1.16

*analytical variation of the amino acid analyses ranged from 6 to 11 %, $N=1$; HAA=hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, methionine and cysteine), PCAA= positively charged amino acids (arginine, histidine and lysine), NCAA=negatively charged amino acids (aspartic+asparagine, glutamic+glutamine, threonine and serine), AAA=aromatic amino acids (phenylalanine and tyrosine), SAA=sulfur-containing amino acids (methionine and cysteine)

drolisis of both hydrolysates GI digestion contributed to higher antioxidant effect. Further hydrolysis was confirmed by measuring the degree of hydrolysis occurring during GI digestion of cod and chicken protein hydrolysates, which showed 2.6- and 2.8-fold higher content of free amino groups in both hydrolysates, respectively, after digestion than in undigested samples (data not shown). Namely, antioxidant ability of peptides depends on peptide size, its amino acid composition and presence of free amino acids within the hydrolysate (3). Similarly, You *et al.* (13), Zhu *et al.* (25), Nalinanon *et al.* (26) and Teixeira *et al.* (27) simulated the process of human GI digestion to determine the changes in antioxidant activities of different fish species. Results showed that GI digestion in general increased their antioxidant properties measured by different *in vitro* assays. In contrast, Borawska *et al.* (28) showed that high degree of hydrolysis of carp muscle tissue led to products with lower free radical scavenging activity.

Additionally, we measured cellular metabolic energy in the cells exposed to digested and undigested hydrolysates, where no significant differences between cod and chicken samples or between digested and undigested samples were observed (Fig. 2), indicating also no cytotoxic effects of hydrolysates on yeast cells before and after digestion.

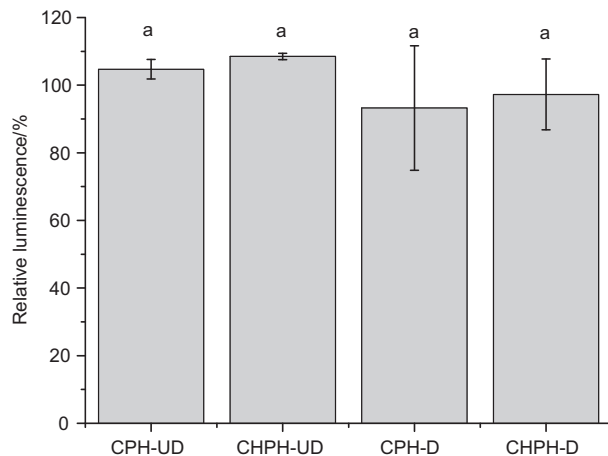


Fig. 2. Cell energy metabolic activity of yeast *Saccharomyces cerevisiae* exposed to undigested and digested cod (CPH-UD and CPH-D, respectively) and chicken protein hydrolysates (CHPH-UD and CHPH-D, respectively). Results are expressed as average values of relative luminescence \pm S.D., $N=3$. Values followed by the same letter are not statistically significantly different ($p \leq 0.05$), as measured by Duncan's test

Proteome changes after exposing yeast cells to digested and undigested cod and chicken protein hydrolysates

To study the effects of protein hydrolysates at a proteome level, before and after GI digestion, the protein

profile of yeast cells treated with both, digested and undigested protein hydrolysates was analyzed. In our previous work, yeast has already been shown to be a good model to investigate the effects of bioactive compounds at a proteome level (11,29).

Proteins from yeast cells exposed to either chicken or cod protein hydrolysates before and after *in vitro* GI digestion were analyzed by 2-D electrophoresis followed by mass spectrometry to identify differentially expressed proteins. Mostly downregulation of proteins was observed compared to the corresponding blanks. In the cells exposed to digested and undigested chicken or cod protein hydrolysates, yeast proteins down-regulated by hydrolysates (fold change >1.50 , $p \leq 0.05$) were identified as peptidyl-prolyl *cis-trans* isomerase (PPIase), elongation factor 1-beta (EF1B), elongation factor 2 (EF2), translationally-controlled tumour protein homologue (Tma19), peroxiredoxin (Tsa1) and cytochrome c oxidase subunit 6 (COX6) (Table 2 and Fig. 3). Additionally, proteins such as EF1B and Tma19 were identified in two spots reflecting different isoforms, post-translational modifications or alternative mRNA splice forms, which could be related to their regulation. Furthermore, exposure of cells to both digested and undigested chicken and cod hydrolysates induced two proteins Bmh1 and Bmh2, which were absent from untreated cells (Table 2 and Fig. 3).

By comparing fold change before and after digestion, it was concluded that digests had greater effect on protein abundance. In contrast to measuring antioxidant activity, where no differences between cod and chicken hydrolysates were observed, here at proteome level, somewhat stronger effect was observed in cod. Since the targets are also proteins related to oxidative stress response, this difference in cellular response to cod and chicken digests at proteome level could be connected to higher content of two amino acids, serine and methionine, in cod than in chicken protein hydrolysates.

Table 2. Fold changes of expression relative to corresponding control (water or digestive juice) of proteins after 2-hour exposure of yeast cells to cod and chicken protein hydrolysates

Spot no.	Fold change relative to the corresponding control				Total score (number of matched peptides)	Protein name/ Acc. no. in NCBI
	CHPH-UD	CPH-UD	CHPH-D	CPH-D		
1	-1.81	-1.10	-1.76	-1.78*	158 (6)	PPIase/gi151941057
2	-1.49	-1.63	-1.19*	-1.88*	217 (7)	EF2/gi6320593
3	-1.49	-1.49	-1.86*	-1.75*	173 (10)	EF1B/gi6319315
4	-1.97	-1.43	-2.09	-1.59*	157 (4)	EF1B/gi6319315
5	-2.69	-2.54	-1.51*	-3.09*	143 (7)	Tma19/gi6322794
6	-1.43	-1.48	-1.30	-1.77*	208 (9)	Tma19/gi6322794
7	-2.36	-1.53	-2.25	-1.92*	238 (4)	Tsa1/gi6323613
8	-1.46	-2.09	-1.94*	-1.86*	118 (4)	Cox6/gi6321842
9	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	181 (7)	Bmh2/683696
10	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	219 (5)	Bmh1/gi671634

*statistically significantly different ($p \leq 0.05$) fold changes of particular proteins between undigested and digested samples (cod and chicken) measured by Student's *t*-test ($p \leq 0.05$)

CPH-UD=cod protein hydrolysate before digestion, CHPH-UD=chicken protein hydrolysate before digestion, CPH-D=cod protein hydrolysate after digestion, CHPH-D=chicken protein hydrolysate after digestion; NCBI=National Center for Biotechnology Information, Bethesda, MD, USA

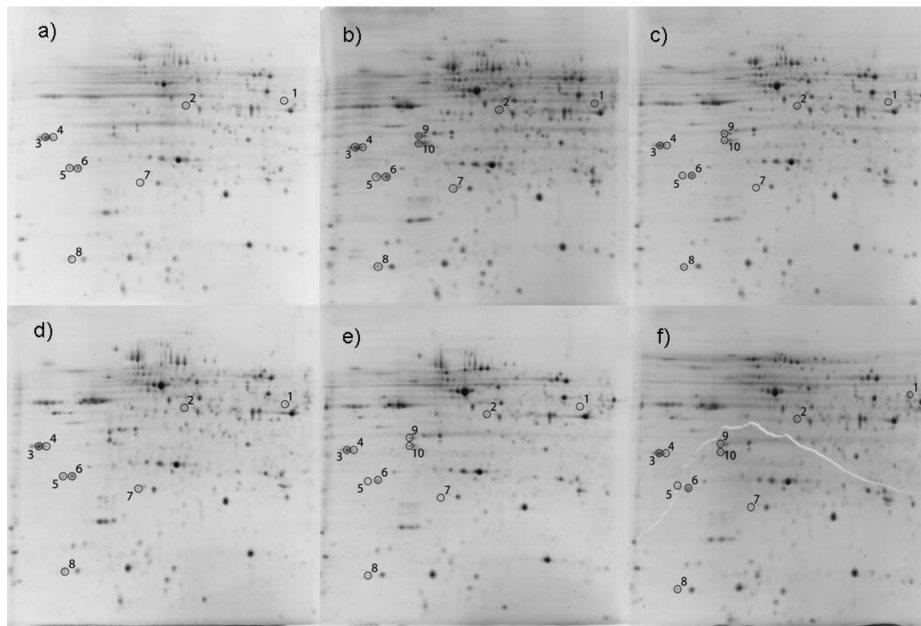


Fig. 3. Representative protein profiles of yeast cells exposed to: a) control (water), b) undigested cod protein hydrolysate, c) undigested chicken protein hydrolysate, d) control (digestive juice), e) digested cod protein hydrolysate, and f) digested chicken protein hydrolysate. Differentially expressed proteins are circled. Details of indicated spots are listed in Table 2. For each gel: from left to right pI=4–7, respectively and from top to bottom $M=220$ –10 kDa, respectively

Function of identified proteins

Elongation factor 1-beta (EF1B) and elongation factor 2 (EF2) belong to proteins related to protein synthesis. EF1B is a subunit of the EF1 complex and a highly conserved protein that has a major role in elongation regulation by regenerating a guanosine triphosphate (GTP)-bound EF1- α , necessary for each elongation cycle. Control at the level of EF1 can modulate the general rate of protein synthesis (30). Eukaryotic elongation factor 2 (eEF2) stimulates the GTP-dependent translocation of the nascent protein chain on the ribosome from the A- to the P-site. There are different factors that control the peptide chain elongation in eukaryotic cell by inhibiting or activating eEF2 (31). The elongation stage of protein synthesis normally consumes a great deal of energy and amino acids (31). It is well established that yeast cells adapt to oxidative stress conditions by changing general gene expression patterns, including transcription and translation of genes related to antioxidants and other stress-induced protective mechanisms. There are many highly abundant proteins involved in oxidative stress response to enable cells to cope with high reactive oxygen species (ROS) level and survive (32). In the cells treated with peptides, a decrease in oxidant level was determined, which means that peptides with antioxidant activity might take part in maintenance of oxidative resistance. Thus, cells might slow down the synthesis of oxidative stress response proteins *via* downregulation of eEF and thus conserve energy. Additionally, Olarewaju *et al.* (33) reported that eEF1B plays a significant role in the oxidative stress response. Namely, deletion of two genes encoding eEF1B γ (subunit of EF1B) in *S. cerevisiae* gave resistance to oxidative stress. Additional roles for eEF1 complex outside the translation system show a tendency for further studies and thus understanding their biological relevance (34).

On the other hand, there are reports suggesting that the machinery of protein synthesis may provide targets for anticancer drugs, since aberrations in protein synthesis (*e.g.* overexpression of translation factors) are commonly encountered in established cancers (35). Thus testing our protein hydrolysates in the context of elongation factor downregulation in different cancer cell lines could be interesting.

Cod and chicken protein hydrolysates before and after digestion caused downregulation of Tma19 protein. Tma19 is the yeast orthologue of mammalian translationally controlled tumour protein (TCTP). TCTP is an evolutionarily highly conserved protein, it shows about 50 % amino acid sequence identity with its most distantly related orthologues in higher organisms (36). Rinnerthaler *et al.* (36) showed that yeast orthologue of TCTP, Tma19, after a mild oxidative stress and various other stress factors is translocated from cytosol to the outer surface of the mitochondria. A stress-induced upregulation of TCTP expression has been reported in many organisms under different stress conditions such as oxidative stress (37) or exposure to heavy metals (38). Based on the data obtained by Rinnerthaler *et al.* (36), the downregulation of Tma19 in our study could mean its transfer from mitochondria back to cytosol, since decreased intracellular oxidation is present in the yeast cells exposed to cod and chicken peptides, especially their digests. Like in our study, TCTP was the target in resveratrol-treated MCF-7 breast cancer cells, where also downregulation was observed (39).

Decrease in intracellular oxidation after exposure to cod and chicken peptides is reflected also on protein Tsa1, which plays an important role in oxidative stress response and redox homeostasis and whose expression decreased. Similarly, expression of protein peptidyl-prolyl *cis-trans* isomerase (PPIase), which is involved in protein refolding

and thus might be indirectly connected to oxidative stress, decreased. Additionally, cytochrome c oxidase subunit 6 was downregulated. This is a subunit of cytochrome c oxidase (COX) or complex IV of the mitochondrial respiratory chain, which has a fundamental role in energy production of aerobic cells. This multimeric enzyme catalyzes the transfer of electrons from cytochrome c to molecular oxygen. Eukaryotic COX is formed by 11–13 subunits (11 in the yeast *Saccharomyces cerevisiae* and 13 in *Homo sapiens*) of dual genetic origin. The assembly of COX made of subunits is a highly regulated process. The regulation involves the availability of subunits and assembly factors regulated at the transcriptional and translational levels, availability of cofactors, protein import into mitochondria and membrane insertion, as well as coordination of sequential or simultaneous steps of the process (40). Therefore, bioactive peptides could be an additional factor in regulation. There are already some studies indicating that antioxidants such as flavonoids can modulate respiratory chain components and inhibit hydrogen production (41). Since antioxidant activity in the cells treated with digested cod and chicken protein hydrolysates increased (Fig. 1), there are no indications showing inhibition of respiration due to downregulation of COX6. Additionally, none of the protein hydrolysates changed cellular metabolic energy in the yeast cells (Fig. 2).

Bmh1 and Bmh2 proteins whose abundance was observed only in treated yeast cells (Table 2), irrespective of the treatment with digested or undigested hydrolysates from cod or chicken, belong to the 14-3-3 protein family, which is highly conserved and has been found in all investigated eukaryotes. They are involved in many different cellular processes, and interact with hundreds of other proteins (42). Therefore, it is difficult to explain precisely their abundance in the treated yeast cells and further studies are needed.

Conclusion

This study revealed that *in vitro* GI digestion of protein hydrolysates, both from cod and chicken muscle, contributed to higher antioxidant activity than in undigested samples. No significant differences in the antioxidant activity were recorded between the two muscle sources, but at proteome level slightly stronger effect was observed of the digests from cod than chicken. Proteins targeted by both hydrolysates belong to different cellular processes (*e.g.* oxidative stress response, protein folding, protein synthesis) and knowledge of their identity is important also in the context of quality and safety of chicken and cod protein hydrolysates as potential functional ingredients. Since we used yeast in the stationary phase, where yeast cells resemble those of multicellular organisms, our results present a good basis for further validation of the activity of cod and chicken protein hydrolysates in higher eukaryotes. This is important for development of new products with chicken and cod protein-derived peptides as functional ingredients.

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References

1. Shahidi F, Zhong Y. Bioactive Peptides. *J AOAC Int.* 2008;91:914–31.
2. Cheung RCF, Ng TB, Wong JH. Marine peptides: bioactivities and applications. *Mar Drugs.* 2015;13:4006–43. <https://doi.org/10.3390/md13074006>
3. Ryan JT, Ross RP, Bolton D, Fitzgerald GF, Stanton C. Bioactive peptides from muscle sources: meat and fish. *Nutrients.* 2011;3:765–91. <https://doi.org/10.3390/nu3090765>
4. Longo VD, Gralla EB, Valentine JS. Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*: mitochondrial production of toxic oxygen species *in vivo*. *J Biol Chem.* 1996;271:12275–80. <https://doi.org/10.1074/jbc.271.21.12275>
5. Kristinsson HG, Theodore AE, Demir N, Ingadottir B. A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. *J Food Sci.* 2005;70:C298–306. <https://doi.org/10.1111/j.1365-2621.2005.tb07177.x>
6. Jónsdóttir R, Geirsdóttir M, Hamaguchi PY, Jamnik P, Kristinsson HG, Undeland I. The ability of *in vitro* antioxidant assays to predict the efficiency of a cod protein hydrolysate and brown seaweed extract to prevent oxidation in marine food model systems. *J Sci Food Agric.* 2016;96:2125–35. <https://doi.org/10.1002/jsfa.7328>
7. ISO 13903:2005. Animal feeding stuffs – Determination of amino acids content. Geneva, Switzerland: International Organization for Standardization (ISO); 2005.
8. Tibäck EA, Svelander CA, Colle IJP, Altskär AI, Alminger MAG, Hendrickx MEG, et al. Mechanical and thermal pretreatments of crushed tomatoes: effects on consistency and *in vitro* accessibility of lycopene. *J Food Sci.* 2009;74:E386–95. <https://doi.org/10.1111/j.1750-3841.2009.01255.x>
9. Cigut T, Polak T, Gašperlin L, Raspor P, Jamnik P. Antioxidative activity of propolis extract in yeast cells. *J Agric Food Chem.* 2011;59:11449–55. <https://doi.org/10.1021/jf2022258>
10. Jakubowski W, Bartosz G. Estimation of oxidative stress in *Saccharomyces cerevisiae* with fluorescent probes. *Int J Biochem Cell Biol.* 1997;29:1297–301. [https://doi.org/10.1016/S1357-2725\(97\)00056-3](https://doi.org/10.1016/S1357-2725(97)00056-3)
11. Görg A. Two-dimensional electrophoresis. *Nature.* 1991;349:545–6.
12. Larsson K, Istenič K, Wulff T, Jónsdóttir R, Kristinsson H, Freysdóttir J, et al. Effect of *in vitro* digested cod liver oil of different quality on oxidative, proteomic and inflammatory responses in the yeast *Saccharomyces cerevisiae* and human monocyte-derived dendritic cells. *J Sci Food Agric.* 2015;95:3096–106. <https://doi.org/10.1002/jsfa.7046>
13. You L, Zhao M, Regenstein JM, Ren J. Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. *Food Chem.* 2010;120:810–6. <https://doi.org/10.1016/j.foodchem.2009.11.018>
14. Nazeer RA, Sampath Kumar NS, Jaiganesh R. *In vitro* and *in vivo* studies on the antioxidant activity of fish peptide isolated from the croaker (*Otolithes ruber*) muscle protein hydrolysate. *Peptides.* 2012;35:261–8. <https://doi.org/10.1016/j.peptides.2012.03.028>
15. Sampath Kumar NS, Nazeer RA, Jaiganesh R. Purification and identification of antioxidant peptides from the skin pro-

- tein hydrolysate of two marine fishes, horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). *Amino Acids*. 2012;42:1641–9.
<https://doi.org/10.1007/s00726-011-0858-6>
16. Bougateg A, Nedjar-Arroume N, Manni L, Ravallec R, Barkia A, Guillochon D, Nasri M. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chem.* 2010;118:559–65.
<https://doi.org/10.1016/j.foodchem.2009.05.021>
 17. Sabeena Farvin KH, Lystbæk Andersen L, Otte J, Nielsen HH, Jessen F, Jacobsen C. Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: fractionation and characterization of peptide fractions. *Food Chem.* 2016;204:409–19.
<https://doi.org/10.1016/j.foodchem.2016.02.145>
 18. Sun Y, Pan D, Guo Y, Li J. Purification of chicken breast protein hydrolysate and analysis of its antioxidant activity. *Food Chem Toxicol.* 2012;50:3397–404.
<https://doi.org/10.1016/j.fct.2012.07>
 19. Onuh JO, Girgih AT, Aluko RE, Aliani M. In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chem.* 2014;150:366–73.
<https://doi.org/10.1016/j.foodchem.2013.10.107>
 20. Centenaro GS, Salas-Mellado M, Pires C, Batista I, Nunes ML, Prentice C. Fractionation of protein hydrolysates of fish and chicken using membrane ultrafiltration: investigation of antioxidant activity. *Appl Biochem Biotechnol.* 2014;172:2877–93.
<https://doi.org/10.1007/s12010-014-0732-6>
 21. Petelinc T, Polak T, Demšar L, Jamnik P. Fractionation of phenolic compounds extracted from propolis and their activity in the yeast *Saccharomyces cerevisiae*. *PLoS One.* 2013;8:e56104.
<https://doi.org/10.1371/journal.pone.0056104>
 22. Samaranayaka AGP, Li-Chan ECY. Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *J Funct Foods.* 2011;3:229–54.
<https://doi.org/10.1016/j.jff.2011.05.006>
 23. Levine RL, Mosoni L, Berlett BS, Stadtman ER. Methionine residues as endogenous antioxidants in proteins. *Proc Natl Acad Sci USA.* 1996;93:15036–40.
<https://doi.org/10.1073/pnas.93.26.15036>
 24. Maralani MN, Movahedian A, Javanmard SH. Antioxidant and cytoprotective effects of L-serine on human endothelial cells. *Res Pharm Sci.* 2012;7:209–15.
 25. Zhu L, Chen J, Tang X, Xiong YL. Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate. *J Agric Food Chem.* 2008;56:2714–21.
<https://doi.org/10.1021/jf703697e>
 26. Nalinanon S, Benjakul S, Kishimura H, Shahidi F. Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chem.* 2001;124:1354–62.
<https://doi.org/10.1016/j.foodchem.2010.07.089>
 27. Teixeira B, Pires C, Nunes ML, Batista I. Effect of in vitro gastrointestinal digestion on the antioxidant activity of protein hydrolysates prepared from Cape hake by-products. *Int J Food Sci Tech.* 2016;51:2528–36.
<https://doi.org/10.1111/ijfs.13233>
 28. Borawska J, Darewicz M, Vegarud GE, Iwaniak A, Minkiewicz P. Ex vivo digestion of carp muscle tissue – ACE inhibitory and antioxidant activities of the obtained hydrolysates. *Food Funct.* 2015;6:210–7.
<https://doi.org/10.1039/C4FO00621F>
 29. Petelinc T, Polak T, Jamnik P. Insight into the molecular mechanisms of propolis activity using a subcellular proteomic approach. *J Agric Food Chem.* 2013;61:11502–10.
<https://doi.org/10.1021/jf4042003>
 30. Venema RC, Peters HI, Traugh JA. Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. *J Biol Chem.* 1991;266:11993–8.
 31. Kaul G, Pattan G, Rafeequi T. Eukaryotic elongation factor-2 (eEF2): its regulation and peptide chain elongation. *Cell Biochem Funct.* 2011;29:227–34.
<https://doi.org/10.1002/cbf.1740>
 32. Morano KA, Grant CM, Moye-Rowley WS. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics.* 2012;190:1157–95.
<https://doi.org/10.1534/genetics.111.128033>
 33. Olarewaju O, Ortiz PA, Chowdhury WQ, Chatterjee I, Kinzy TG. The translation elongation factor eEF1B plays a role in the oxidative stress response pathway. *RNA Biol.* 2004;1:89–94.
<https://doi.org/10.4161/rna.1.2.1033>
 34. Sasikumar AN, Perez WB, Kinzy TG. The many roles of the eukaryotic elongation factor 1 complex. *WIREs RNA.* 2012;3:543–55.
<https://doi.org/10.1002/wrna.1118>
 35. Arora S, Yang JM, Kinzy TG, Utsumi R, Okamoto T, Kitayama T, et al. Identification and characterization of an inhibitor of eukaryotic elongation factor 2 kinase against human cancer cell lines. *Cancer Res.* 2003;63:6894–9.
 36. Rinnerthaler M, Jarolim S, Heeren G, Palle E, Perju S, Klinger H, et al. MMI1 (YKL056c, TMA19), the yeast orthologue of the translationally controlled tumor protein (TCTP) has apoptotic functions and interacts with both microtubules and mitochondria. *BBA – Bioenergetics.* 2006;1757:631–8.
<https://doi.org/10.1016/j.bbabi.2006.05.022>
 37. Yan Y, Weaver VM, Blair IA. Analysis of protein expression during oxidative stress in breast epithelial cells using a stable isotope labeled proteome internal standard. *J Proteome Res.* 2005;4:2007–14.
<https://doi.org/10.1021/pr050175d>
 38. Schmidt I, Föhling M, Nafz B, Skalweit A, Thiele BJ. Induction of translationally controlled tumor protein (TCTP) by transcriptional and post-transcriptional mechanisms. *FEBS J.* 2007;274:5416–24.
<https://doi.org/10.1111/j.1742-4658.2007.06069.x>
 39. Díaz-Chávez J, Fonseca-Sánchez MA, Arechaga-Ocampo E, Flores-Pérez A, Palacios-Rodríguez Y, Domínguez-Gómez G, et al. Proteomic profiling reveals that resveratrol inhibits HSP27 expression and sensitizes breast cancer cells to doxorubicin therapy. *PLoS One.* 2013;8:e64378.
<https://doi.org/10.1371/journal.pone.0064378>
 40. Fontanesi F, Soto IC, Horn D, Barrientos A. Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process. *Am J Physiol Cell Physiol.* 2006;291:C1129–47.
<https://doi.org/10.1152/ajpcell.00233.2006>
 41. Lagoa R, Graziani I, Lopez-Sanchez C, Garcia-Martinez V, Gutierrez-Merino C. Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. *BBA – Bioenergetics.* 2011;1807:1562–72.
<https://doi.org/10.1016/j.bbabi.2011.09.022>
 42. van Heusden GPH, Steensma HY. Yeast 14-3-3 proteins. *Yeast.* 2006;23:159–71.
<https://doi.org/10.1002/yea.1338>