

Carp Proteins as a Source of Bioactive Peptides – an *in Silico* Approach

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Abstract

DAREWICZ M., BORAWSKA J., PLISZKA M. (2016): **Carp proteins as a source of bioactive peptides – an *in silico* approach.** Czech J. Food Sci., 34: 111–117.

In silico prediction methods and tools were used to determine whether bioactive peptides are hidden in the amino acid sequences of carp (*Cyprinus carpio*) proteins and whether they can be released after gastric, duodenal, and gastro-duodenal *in silico* digestion. Fragments with 15 types of biological activity in intact proteins and, after gastrointestinal digestion, with 11 types of activity were identified. Angiotensin I-converting enzyme inhibitory (ACEi) and antioxidant peptides were the most predominant fragments of proteins in the group of intact proteins and *in silico* digests. After *in silico* digestion the highest number of sequences with ACEi activity (108) and antioxidant activity (13) was released after gastro-duodenal digestion of the myosin heavy chain.

Keywords: biological activity; bioinformatics; BIOPEP; fish; databases; proteolysis

Food proteins are a source of bioactive peptides that are capable of regulating body functions, and those properties are used in the production of functional foods (BLEIEL 2010). MINKIEWICZ *et al.* (2008a) proposed the concept of a food peptidome as the resource of all peptides in unprocessed and processed foods. Biopeptides are released from food protein precursors during hydrolysis with endogenous or exogenous enzymes, fermentation and maturation, chemical synthesis or through the expression of corresponding genes (SAADI *et al.* 2015; ZAMBROWICZ *et al.* 2015). Fish proteins are a potential source of biopeptides. A number of previous studies have described the biological activity of marine protein hydrolysates produced from different species (RYAN *et al.* 2011; IWANIAK *et al.* 2014). Carp (*Cyprinus carpio* L.) is one of the most popular aquaculture fishes in Europe whose meat contains 15–18% of protein (SKIBNIEWSKA *et al.* 2013). The leading European suppliers of carp are Germany, the Czech Republic, Slovakia, Hun-

gary, Lithuania, and Poland (European Commission 2014). Research into food proteins and peptides increasingly often relies on *in silico* methods and tools. One of the most popular *in silico* tools is BIOPEP – the database of bioactive food proteins and peptides (<http://uwm.edu.pl/biochemia>). LI-CHAN (2015) in a review article relied on BIOPEP as a standard source of information about bioactive food peptides, whereas MARTÍNEZ-ALVAREZ (2013) deployed the discussed database to search for information about seafood biopeptides. DAREWICZ *et al.* (2014) applied the above database and mass spectrometry (MS) to identify peptides with Angiotensin I-converting enzyme inhibitor (ACEi) activity in products of salmon protein hydrolysis/digestion. CAPRIOTTI *et al.* (2015) used the sequences of peptides identified by MS in fish protein hydrolysates to make queries in CAMP (<http://www.bicnirrh.res.in/antimicrobial>) and BIOPEP databases. TOOPCHAM *et al.* (2015) used the peptides identified in tilapia protein hydrolysates as BIOPEP queries. To the

Supported by the National Science Centre in Poland, Project No. N N312 465240, and by the University of Warmia and Mazury in Olsztyn, Poland.

best of our knowledge, the potential of selected carp proteins to generate biopeptides via the human gastrointestinal tract has not been investigated to date. Such circumstances encouraged us to undertake the study aimed at identifying potential precursors of bioactive peptides in carp proteins and predicting whether such biopeptides can be released during gastrointestinal digestion in humans.

MATERIAL AND METHODS

Material. The amino acid sequences of carp proteins retrieved from the UniProtKB database (<http://www.uniprot.org/>) (The UniProt Consortium 2015) were studied. Identical sequences were eliminated using the ClustalW2 – Multiple Sequence Alignment program with default settings (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) (LARKIN *et al.* 2007). A total of 33 amino acid sequences of carp proteins with less than 90% identity were selected for further analyses (Table 1). Sequences of biopeptides were obtained from BIOPEP. The database contained amino acid sequences of 2609 biopeptides with 48 types of activity determined *in vitro* and *in vivo* (accessed in November 2014) (MINKIEWICZ *et al.* 2008a).

Methods. The profiles of potential biological activity were used to define the type and location of bioactive fragments in the protein molecule. Parameter *A*, defined as the frequency of occurrence of bioactive fragments in a protein sequence, and parameter *B*, defined as potential ACEi activity of proteins (MINKIEWICZ *et al.* 2008b), were used according to the following formula:

$$A = a/N \quad (1)$$

where:

a – number of fragments with specific bioactivity in a protein sequence

N – number of amino acid residues of protein

$$B_i = \frac{\sum_i^k \frac{a_i}{IC_{50i}}}{N} \quad (2)$$

where:

a_i – number of repetitions of the *i*th ACEi fragment in a protein sequence

IC_{50i} – concentration of the *i*th ACEi peptide corresponding to its half-maximal activity (μM)

k – number of different fragments with ACEi activity

N – number of amino acid residues in protein

Parameter *B* was determined only for ACEi activity due to the lack of *IC₅₀* values for some fragments with other types of bioactivities.

To predict the possibility of enzymatic release of biopeptides, computational tools in BIOPEP were used to simulate gastric digestion by pepsin (EC 3.4.23.1), duodenal digestion simulation by trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), and gastro-duodenal digestion by pepsin, followed by trypsin and chymotrypsin.

In the hydrolysates, the frequency (*A_E*) and relative frequency (*W*) with which bioactive fragments were released by selected enzymes from protein sequences were determined according to the following formulas (MINKIEWICZ *et al.* 2011):

$$A_E = d/N \quad (3)$$

where:

d – number of fragments with specific bioactivity in a protein sequence that can be released by enzyme/s

N – number of amino acid residues of protein

$$W = A_E/A \quad (4)$$

where:

A_E – frequency of release of fragments with given activity by selected enzymes

A – frequency of occurrence of bioactive fragments in a protein sequence

RESULTS AND DISCUSSION

Profiles of potential biological activity, frequency of occurrence of bioactive fragments and potential biological activity of carp proteins. The number of amino acid residues of the analysed amino acid sequences of carp proteins ranged from 62 (light meromyosin, Q90335) to 1938 (myosin heavy chain, Q2HX56) (Table 1). Biological activity profiles supported the identification of fragments with 15 types of activity: ACEi, antioxidant, anti-amnesic, antithrombotic, immunomodulatory, inhibiting dipeptidyl peptidase IV, activating ubiquitin-mediated proteolysis, bacterial permease ligand, chemotactic, anorectic, opioid, anti-carcinogenic, regulating ion flow, regulating the stomach mucosal membrane activity, and the phosphoinositide metabolisms. The highest number of 933 biopeptides was determined in the myosin sequences, and the lowest number of 32 biopeptides – in light meromyosin (Table 2). ACEi and antioxidant peptides were hidden

doi: 10.17221/300/2015-CJFS

Table 1. Carp (*Cyprinus carpio*) proteins examined in the *in silico* study

Protein	Accession number (UniProtKB)	Molecular mass (kDa)	Chain length of protein without signal peptide
Miofibrillar proteins			
Actin-alpha, skeletal muscle	P53479	41.959	377
Actin-beta	P83750	41.753	375
Skeletal muscle actin mutant	Q6TKP4	41.975	377
Skeletal muscle alpha-actin	Q6TKP5	41.929	377
Skeletal muscle actin (Fragment)	Q7T2J3	41.961	377
Myosin heavy chain	Q90339	221.601	1937
Myosin heavy chain embryonic type 2	Q2HX57	221.895	1935
Myosin heavy chain embryonic type 3	Q2HX56	222.543	1938
Myosin heavy chain	Q5NTZ3	220.988	1931
Myosin heavy chain	Q90337	221.093	1933
Myosin heavy chain	O42352	221.163	1931
Myosin heavy chain embryonic type 1	Q2HX58	221.683	1932
Fast skeletal myosin light chain 1a	Q90331	21.128	193
Fast skeletal myosin light chain 1b	Q90332	21.134	193
Fast skeletal myosin light chain 3	Q90333	16.805	151
Myosin regulatory light chain	Q9I892	18.896	169
Light meromyosin (Fragment)	Q90335	6.941	62
Sarcoplasmic proteins			
Parvalbumin-alpha	P09227	11.451	108
Parvalbumin-beta	P02618	11.436	108
Parvalbumin, cyp c 1.01	Q8UUS3	11.504	109
Parvalbumin, cyp c 1.02	Q8UUS2	11.569	109
Myoglobin	P02204	15.776	147
Myoglobin isoform 2	Q2LC33	16.174	147
Alpha globin type-2	Q8UW95	15.800	143
Alpha globin type-2	Q8UW92	15.757	144
Alpha globin type-2	O13135	15.431	143
Alpha globin type-2	Q8UW94	16.706	147
Beta globin type-3	Q8UW93	16.375	147
Beta-globin	O13140	16.343	148
The other (rest) proteins			
Hemoglobin subunit alpha (Hemoglobin alpha chain)	P02016	15.447	143
Hemoglobin subunit beta-A/B (Hemoglobin beta-A/B chain)	P02139	16.262	143
Heat shock protein 4	Q7ZZH6	94.463	841
Muscle-specific heat shock protein Hsc70-1 (Fragment)	Q7T276	70.362	639

in the highest number of fragments per each analysed protein molecule. Myofibrillar proteins, in particular myosin heavy chains, were the potentially richest source of bioactive fragments, including ACEi (646)

and antioxidant peptides (120). Food ACEi peptides have been comprehensively studied as natural compounds for the treatment of hypertension (IWANIAK *et al.* 2014). The predominant peptides with ACEi

Table 2. Maximum and minimum numbers (a) of potential bioactive peptides (per protein molecule) identified in the carp (*Cyprinus carpio*) proteins, and A^* and B^* values

	Miofibrillar proteins			Sarcoplasmic proteins			The other (rest) proteins		
	a	A	B (μM^{-1})	a	A	B (μM^{-1})	a	A	B (μM^{-1})
Bioactive peptides	max	933	0.6321	106	0.7273	nd	401	0.7063	nd
	min	32	0.4503	60	0.5139	nd	99	0.4768	nd
ACEi peptides	max	646	0.4111	73	0.4966	0.0340	286	0.4336	0.0338
	min	23	0.3271	42	0.2917	0.0053	59	0.2762	0.0055
Antioxidant peptides	max	120	0.0619	16	0.1088	nd	44	0.0769	nd
	min	2	0.0323	3	0.0275	nd	11	0.0391	nd

*parameter A is the frequency of occurrence of bioactive fragments in the protein and parameter B is defined as potential biological activity of protein; nd – not determined due to the lack of IC_{50} values for all antioxidative fragments found in the amino acid sequence of protein

activity were dipeptides and tripeptides. The small peptides are known to be more potent ACE inhibitors than the larger peptides, probably because they better fit into an ACE active site (IWANIAK *et al.* 2014). Tripeptides were predominant antioxidant peptides in the identified sequences. Antioxidant peptides generally comprise 3 to 16 amino acid residues (SAADI *et al.* 2015). Alpha-globin was characterised by the highest frequency of occurrence of all bioactive sequences ($A = 0.7273$), whereas the highest frequency of occurrence of ACEi and antioxidant peptides was noted in myoglobin ($A = 0.4966$ and 0.1088 , respectively) (Table 2). The myosin heavy chain was characterised by the highest potential ACEi activity ($B = 0.0592 \mu\text{M}^{-1}$). The carp myofibrillar

proteins containing more amino acid residues than the sarcoplasmic proteins are an example which confirms the rule that the longer the protein sequence, the greater the chance of finding a bioactive fragment. Similar conclusions were formulated by MINKIEWICZ *et al.* (2011) in an *in silico* study of beef proteins.

Potential bioactive peptides predicted to be released per protein molecule, frequency and relative frequency of the release of fragments with ACEi and antioxidative activity after gastric, duodenal, and gastro-duodenal *in silico* digestion. The highest number of biopeptides per protein molecule was predicted to be released from the myosin heavy chain after *in silico* simulation of gastric, duodenal,

doi: 10.17221/300/2015-CJFS

and gastro-duodenal digestion at 144, 125, and 139, respectively (data not shown). Peptides released during proteolysis of carp proteins were characterised by 11 types of activity: ACEi, antioxidant, enzyme inhibiting (other than ACE), ion flow regulating, hypotensive, antithrombotic, activating ubiquitin-mediated proteolysis, immunomodulatory, glucose uptake stimulating, anti-amnesic, and of bacterial permease ligand. A_E and W of release were calculated for the predominant ACEi and antioxidant peptides (Table 3). As a result of gastric, duodenal, and gastro-duodenal digestion, the highest number of ACEi and antioxidant peptides was predicted to be released from the myosin heavy chain at 105, 56, and 108 for ACEi and 11, 18, and 13 for antioxidant peptides, respectively. The highest A_E of ACEi peptide release was noted after gastric digestion of carp myoglobin at 0.0952. A_E values decreased in

the successive stages of digestion. The frequency of antioxidant peptide release was the highest after gastro-duodenal digestion of alpha-globin at 0.0210. In turn, W of the release of ACEi peptides was the highest (0.1961) in the carp myosin light chain after gastric digestion, whereas the highest value of W (0.2727) for antioxidant peptides was noted in alpha-globin after gastro-duodenal digestion. After digestion, myofibrillar proteins appeared as a richer source of ACEi and antioxidant peptides than sarcoplasmic proteins (Table 2, parameter d).

Hypothetically, the richer the profile of biological activity of a protein molecule (Table 1, parameter A), the greater the probability of biopeptide release (MINKIEWICZ *et al.* 2011). This theoretical prediction was confirmed in our experiment where the myosin heavy chain contained the highest number of sequences of ACEi and antioxidant peptides. The

Table 3. Number (maximum and minimum) of potential bioactive peptides predicted to be released per protein molecule (d), frequency (A_E) and relative frequency (W) of the release of fragments with ACEi and antioxidative activity after gastric, duodenal, and gastro-duodenal *in silico* digestion of carp (*Cyprinus carpio*) proteins

	ACEi peptides		Antioxidant peptides	
	max	min	max	min
Gastric	d	105 myosin heavy chain (Q5NTZ3)	4 myosin light chain (Q9I892), α -globin (O13135)	11 myosin heavy chain (Q2HX57)
	A_E	0.0952 myoglobin (Q2LC33)	0.0237 myosin light chain (Q9I892)	0.0140 α -globin (Q8UW95), hemoglobin (P02139)
	W	0.2174 light meromyosin (Q90335)	0.0678 myosin light chain (Q9I892)	0.2222 β -globin (O13140)
Duodenal	d	56 myosin heavy chain (Q2HX56)	0 myosin light chain (Q9I892), α -globin (Q8UW92)	18 myosin heavy chain (Q2HX57)
	A_E	0.0463 parvalbumin (P02618)	0 myosin light chain (Q9I892), α -globin (Q8UW92)	0.0204 β -globin (Q8UW94)
	W	0.1042 parvalbumin (P02618)	0.0323 β -globin (Q8UW93)	0.2000 β -globin (Q8UW94)
Gastro-duodenal	d	108 Myosin heavy chain (O42352)	2 Myosin light chain (Q9I892)	13 myosin heavy chain (Q2HX57)
	A_E	0.0748 myoglobin (Q2LC33)	0.0339 myosin light chain (Q9I892)	0.0210 α -globin (Q8UW95)
	W	0.1841 myosin light chain (Q90333)	0.0339 myosin light chain (Q9I892)	0.2727 α -globin (Q8UW95)
				1 α -globin (O13135, Q8UW92), β -globin (Q8UW93), hemoglobin (P02016), myosin light chain (Q90331, Q90332)
				0.0031 heat shock protein (Q7T276)
				0.0667 myosin heavy chain (Q2HX56)

in silico digestion revealed that the myosin heavy chain could be the best source of bioactive sequences (Table 2, parameter d). The probability that bioactive peptides will be released from a given protein is also determined by enzyme specificity. Enzymes with broader specificity contribute to the release of a higher number of biopeptides (Ahn *et al.* 2013). *In silico* digestion of carp proteins led to the release of significantly more ACEi than antioxidant peptides. It should also be noted that the majority of ACEi peptides in the analysed protein sequences contain two amino acids, whereas most antioxidant peptides are composed of three amino acids. There is a greater probability that the released fragments will contain two amino acids rather than three amino acids, especially that the *in silico* efficiency of digestion is expected to reach 100% as confirmed by PAMPANIN *et al.* (2012).

The activity of hydrolysates obtained *in vitro* may differ even if the same substrates and enzymes were used. This is determined by the conditions of protein hydrolysis and protein structure. In view of the above, *in silico* methods can be recommended for screening and pre-selection of protein sequences to predict the potential biological activity of their fragments and to search for new methods of releasing bioactive peptides from precursor sequences.

Our study is a preliminary screening of the potential health-promoting biological activity of carp proteins. As the food components, bioactive peptides from carp proteins might be the valuable agents in the prevention of some diet-related diseases. ACE inhibitory and antioxidant peptides may have a physiological relevance in the regulation of blood pressure and oxidation processes in the human body. Moreover, antioxidant peptides from carp proteins can be used as prevention agents of adverse changes of texture, sensory characteristics, functional features, and nutritional values in food.

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Received: 2015–06–10

Accepted after corrections: 2016–02–26

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