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

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**Abstract**

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*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, Hungary, 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

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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 $IC_{50}$ 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, antiamnesic, antithrombotic, immunomodulatory, inhibiting dipeptidyl peptidase IV, activating ubiquitin-mediated proteolysis, bacterial permease ligand, chemotactic, anorectic, opioid, anticarcinogenic, 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

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

| Protein | Accesion number (UniProtKB) | Molecular mass (kDa) | Chain length of protein without signal peptide |
|---|---|---|---|
| **Miofibryllar 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

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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

| | | Miofibryllar proteins | | | Sarcoplasmic proteins | | | The other (rest) proteins | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | a | *A* | *B* ($\mu M^{-1}$) | a | *A* | *B* ($\mu M^{-1}$) | a | *A* | *B* ($\mu M^{-1}$) |
| Bioactive peptides | max | 933 myosin heavy chain (Q5NTZ3) | 0.6321 myosin light chain (Q90332) | nd | 106 myoglobin (P02204) | 0.7273 α-globin (O13135) | nd | 401 heat shock protein (Q7ZZH6) | 0.7063 hemoglobin subunit (P02139) | nd |
| | min | 32 light meromyosin (Q90335) | 0.4503 myosin light chain (Q90333) | nd | 60 parval-bumin (P09227, Q8UUS2, Q8UUS3) | 0.5139 α-globin (Q8UW92) | nd | 99 hemoglobin subunit (P02016) | 0.4768 heat shock protein (Q7ZZH6) | nd |
| ACEi peptides | max | 646 myosin heavy chain (Q5NTZ3) | 0.4111 aktyna (Q6TKP5) | 0.0592 myosin heavy chain (Q2HX57) | 73 myoglobin (P02204) | 0.4966 myoglobin (P02204) | 0.0340 β-globin (O13140) | 286 heat shock protein (Q7ZZH6) | 0.4336 hemoglobin subunit (P02139) | 0.0338 hemoglobin subunit (P02139) |
| | min | 23 light meromyosin (Q90335) | 0.3271 myosin heavy chain (Q2HX56, Q2HX58) | 0.0029 myosin light chain (Q90333) | 42 α-globin (Q8UW92) | 0.2917 α-globin (Q8UW92) | 0.0053 parvalbu-min (Q8UUS3) | 59 hemoglobin subunit (P02016) | 0.2762 heat shock protein (Q7ZZH6) | 0.0055 heat shock protein (Q7T276) |
| Antioxidant peptides | max | 120 myosin heavy chain (Q2HX56) | 0.0619 myosin heavy chain (Q2HX56) | nd | 16 myoglobin (P02204) | 0.1088 myoglobin (P02204) | nd | 44 heat shock protein (Q7ZZH6) | 0.0769 hemoglobin subunit (P02016, P02139) | nd |
| | min | 2 light meromyosin (Q90335) | 0.0323 light meromyosin (Q90335) | nd | 3 parvalbu-min (P0218, Q8UUS2) | 0.0275 parvalbu-min (Q8UUS2) | nd | 11 hemoglobin subunit (P02016, P02139) | 0.0391 heat shock protein (Q7T276) | 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 $\mu M^{-1}$). 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,

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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, antiamnesic, 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<br>myosin heavy chain (Q5NTZ3) | 4<br>myosin light chain (Q9I892), α-globin (O13135) | 11<br>myosin heavy chain (Q2HX57) | 0<br>α-globin (O13135, Q8UW92), hemoglobin (P02016) |
| | $A_E$ | 0.0952<br>myoglobin (Q2LC33) | 0.0237<br>myosin light chain (Q9I892) | 0.0140<br>α-globin (Q8UW95), hemoglobin (P02139) | |
| | $W$ | 0.2174<br>light meromyosin (Q90335) | 0,0678<br>myosin light chain (Q9I892) | 0,2222<br>β-globin (O13140) | |
| Duodenal | d | 56<br>myosin heavy chain (Q2HX56) | 0<br>myosin light chain (Q9I892), α-globin (Q8UW92) | 18<br>myosin heavy chain (Q2HX57) | 0<br>myosin light chain (Q90331, Q90332) |
| | $A_E$ | 0.0463<br>parvalbumin (P02618) | 0<br>myosin light chain (Q9I892), α-globin (Q8UW92) | 0.0204<br>β-globin (Q8UW94) | |
| | $W$ | 0.1042<br>parvalbumin (P02618) | 0.0323<br>β-globin (Q8UW93) | 0.2000<br>β-globin (Q8UW94) | |
| Gastro-duodenal | d | 108<br>Myosin heavy chain (O42352) | 2<br>Myosin light chain (Q9I892) | 13<br>myosin heavy chain (Q2HX57) | 1<br>α-globin (O13135, Q8UW92), β-globin (Q8UW93), hemoglobin (P02016), myosin light chain (Q90331, Q90332) |
| | $A_E$ | 0.0748<br>myoglobin (Q2LC33) | 0.0339<br>myosin light chain (Q9I892) | 0.0210<br>α-globin (Q8UW95) | 0.0031<br>heat shock protein (Q7T276) |
| | $W$ | 0.1841<br>myosin light chain (Q90333) | 0.0339<br>myosin light chain (Q9I892) | 0.2727<br>α-globin (Q8UW95) | 0.0667<br>myosin heavy chain (Q2HX56) |

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*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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