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Food Protein Fragments Are Regulatory Oligopeptides

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Abstract—Until recently food proteins were considered to be an energy source and a source of essential and nonessential amino acids required for protein synthesis and precursors of many vital biomolecules. However, we assumed earlier that food protein fragments might perform some regulatory functions. The theoretical justification for this assumption is advanced in this work. In the present work, the primary structures of protein fragments were compared with amino acid sequences of known natural regulatory oligopeptides *in silico*. It is shown that fragments formed as a result of animal food protein cleavage by proteolytic enzymes can exist in the gastrointestinal tract for a long time. Many of them are enzyme inhibitors, regulators of nervous, endocrine, and immune system, and possess antimicrobial and other activities. It has also been shown that the lifetime of fragments before their cleavage in the gastrointestinal tract could be enough for performing corrective functions. Thus, as a result of food protein fragmentation a dynamic pool of exogenous regulatory oligopeptides with functions changing as shorter fragments are generated may form. The detection of an endogenous–exogenous pool of regulatory molecules expands the significance and content of the Ashmarin–Obukhova hypothesis on a functional continuum of natural oligopeptides. The possible practical importance of these results is noted.

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Endogenous oligopeptides are known to regulate numerous vital processes in all living organisms [1]. Natural biologically active oligopeptide structures have been identified in more than 2000 species of organisms. More than 10,000 are now known, and the number is growing rapidly [2, 3]. In humans alone there are more than 600 oligopeptide regulators of neural, endocrine, immune system, and many other physiological processes. The variety of oligopeptide structures and functions in a single organism led Ashmarin and Obukhova [4] to formulate the hypothesis of functionally continuous set (a continuum) of endogenous oligopeptides.

In most cases endogenous regulatory peptides are fragments of specialized precursors [5, 6], and their formation is regulated by a number of biochemical processes. Such oligopeptides are found in many organs and tissues including the gastrointestinal tract. This indicates that they are not cleaved by proteases for some time, and they can thus express their physiological activities.

Many facts have become known confirming that in many organs and tissues of living organisms endogenous peptide structures are also present that are not formed from specialized precursors, but are natural fragments of well-known proteins. A large number of physiologically active fragments of hemoglobin α- and β-chains have been identified in bovine brain [7-10] and thymus [11], fragments of casein in cows' milk [12, 13], and other substances. They are formed by cleavage of the precursor molecules by proteases and can also exist and function for some time before final breakdown. Many various proteases coexist in one organism, and their local concentrations are not known in most cases. That is why experimental determination of the lifetime of fragments formed by endogenous peptides remains an unsolved problem.

Along with endogenous proteins, exogenous proteins are also present in a living organism. They enter regularly with food. These proteins are also exposed to proteases in the gastrointestinal tract, resulting in their successive breakdown into smaller and smaller fragments down to amino acids. Until recent time they were viewed mainly

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as one of the sources of energy as well as essential and nonessential amino acids necessary for protein synthesis, as well as serving as precursors for many vital biomolecules [14]. However, we assumed earlier that fragments of food proteins could also carry out regulatory functions [6, 15-17]. This work provides a theoretical basis for this assumption. The first proteolytic enzyme to be encountered by a food protein is pepsin, which is secreted in the stomach [18]. That is why significant attention is given to this enzyme in our work. Proteins present in bovine muscles (actin, myosin, and hemoglobin) and cows' milk (casein) were used as substrates.

METHODS OF INVESTIGATION

Investigations were conducted by comparing primary structures of protein fragments with amino acid sequences of natural regulatory oligopeptides known to date. Primary structures of proteins were taken from the UniProtKB/Swiss-Prot database [19] (http://uniprot.org), and the data on natural regulatory oligopeptides was taken from the specialized EROP-Moscow database (http://erop.inbi.ras.ru). At the time of our investigation it contained data about primary structure and functions of 10,355 natural oligopeptide regulators. Protein fragments were compared with known oligopeptides using a specially created computer program. Full amino acid sequences of proteins that served as a source of fragments of specified length were used as input, and step-by-step fragmenting was performed, as well as comparison of resulting fragments with all oligopeptides in the EROP-Moscow database. For example, in the case of dipeptide fragments this procedure revealed fragments with amino acid numbers from the *N*-terminus (1-2, 2-3, etc.) to the *C*-terminus. The events of concurrence of fragment amino acid sequences with primary structure of a natural oligopeptide were automatically put into the results table. Then this data was processed by standard programs that sorted and selected molecular groups by given structural and functional characteristics.

Then amino acid residues were identified in the protein primary structure from which *N*- and *C*-termini the given fragment was cleaved by pepsin. The ability of pepsin to cleave different peptide bonds is significantly affected by pH [20]. Its maximum activity is observed at pH 1.9. It preserves its activity at pH 1.0 and loses it at pH > 5.0 [21]. We used data about the possibility of cleavage of all possible combination of amino acid residues at pH 1.0, 2.5, and 4.0 by porcine pepsin. Data was obtained *in vitro* on 58 different proteins [22]. By normalizing these data, the possibilities of cleavage of all variants (400) of peptide bonds by pepsin at three pH values were calculated. Repeated participation of the enzyme in substrate cleavage is characterized by its turnover rate, which is 0.5 sec^{-1} for pepsin [23]. This value characterizes the fact that the events of interaction of a single enzyme molecule with substrate molecules occur approximately every 2 sec. In calculation of enzyme concentration, the volume of human stomach was assumed to be 1 liter [24].

Then the probability of accumulation of an uncleaved fragment of the selected protein was calculated. For this, the probabilities of cleavage by pepsin of peptide bonds surrounding the fragment in the protein were calculated, and the probabilities of non-cleavage of peptide bonds inside the fragment. In case of dipeptide fragments a sequence $\mathbf{A_2}$–$\mathbf{A_3}$ of their amino acid residues was examined together with surrounding residues A_1 and A_4: A_1–$\mathbf{A_2}$–$\mathbf{A_3}$–A_4. The probabilities of cleavage of A_1–$\mathbf{A_2}$ and $\mathbf{A_3}$–A_4 peptide bonds are P_{1-2} and P_{3-4}, respectively, and the probability of non-cleavage of the $\mathbf{A_2}$–$\mathbf{A_3}$ bond is $1 - P_{2-3}$. In accordance of the rule of probability for independent events [25], the probability for the $\mathbf{A_2}$–$\mathbf{A_3}$ bond to remain uncleaved after the first ($n = 1$) enzyme actions and for A_1–$\mathbf{A_2}$ and $\mathbf{A_3}$–A_4 bonds to be cleaved is:

$$P_{2\text{-mer}} = P_{1-2}(1 - P_{2-3})P_{3-4}. \quad (1)$$

With the initial protein concentration of C_0, the concentration of the formed dipeptide is:

$$C_1 = C_0P_{2\text{-mer}} = C_0P_{1-2}(1 - P_{2-3})P_{3-4}. \quad (2)$$

In this work only such fragments are analyzed for which the probability of cleavage of the peptide bonds equals zero, that is, $P_{2-3} = 0$ (the more complex case, when $P_{2-3} > 0$, will be analyzed in a separate publication). Therefore, Eq. (2) can be simplified:

$$C_1 = C_0P_{1-2}P_{3-4}. \quad (3)$$

After the first cycle of enzymatic catalysis, the enzyme molecule becomes free from the enzyme–substrate complex and is ready for the next catalytic act.

In the second cycle of enzyme action, the concentration of intact protein is decreased and equals $C_0 - C_1$, and the concentration of formed fragments is:

$$C_2 = (C_0 - C_1)P_{1-2}P_{3-4}. \quad (4)$$

In the third cycle

$$C_3 = (C_0 - C_2)P_{1-2}P_{3-4} \quad (5)$$

and so on till the n-th cleavage

$$C_n = (C_0 - C_{n-1})P_{1-2}P_{3-4}. \quad (6)$$

Summing each new portion of the formed fragment with all previous ones leads to a recurrent formula that expresses the total number of fragments formed after each enzyme cycle:

$$C_{sum} = (C_0 - \sum_{1}^{n-1} C_n)\, P_{1-2}\, P_{3-4} + \sum_{1}^{n-1} C_n. \quad (7)$$

As the accumulation of the resulting fragment is a function of discrete values n, i.e.

$$C_{sum} = f(n), \quad (8)$$

this process is also a function of time, as $t = 1/n$. Therefore,

$$C_{sum} = f(t), \quad (9)$$

where t is also a discrete value. Taking into consideration the duration of one pepsin molecule cycle, calculations were performed for 72,000 cycles, which equals 40 h of enzyme interaction with the substrate. This made it possible to obtain the dependence of C_{sum} on t, representing the kinetic curves of accumulation of fragments.

Proteins of bovine muscles were selected as objects of investigation: actin, myosin (three subunits – heavy MH chain and light MLa and MLr chains), hemoglobin (α- and β-chains), as well as all known cow milk caseins: α-S1, α-S2, β, and κ. For calculations, we used the amount of substances typically consumed by people at one meal. These quantities were assumed as 200 g of beef and 200 ml of milk. The concentration of protein C_0 (Table 1) was calculated from known data for actin and myosin [26], hemoglobin [27], and caseins [28]. Molecular weights of proteins were obtained by subtraction of signal peptides masses from molecular masses of the precursors presented in the UniProtKB/Swiss-Prot database [19].

The concentration of pepsin, as taken for this study, in the human stomach is 349.1 μg/ml [29]. With molecular weight of 40,306 Da, human pepsin has molar concentration C_E of $8.7 \cdot 10^{-6}$ M, which is less than the initial concentration C_0 of the proteins we selected. This means that not all of the substrate molecules can be subject to the action of the enzyme in each cycle. Therefore, the factor $k = C_E/C_0$ was added to our calculations, characterizing the portion of the substrate subjected to the enzyme action. As a result, formula (7) was modified:

$$C_{sum} = (C_0 - \sum_{1}^{n-1} C_n)\, k\, P_{1-2}\, P_{3-4} + \sum_{1}^{n-1} C_n. \quad (7a)$$

Table 1. Protein content in skeletal muscle and milk

UniProt No.	Object of study	Wet weight (volume)	Dry weight		All proteins		Given protein		Mass, Da	C_0, M
			%	g	%	g	%	g		
	Skeletal muscles	200 g	30 [25]	60	60 [25]	36.3				
P68138	actin myosin						13 [25] 29 [25]	4.72 10.53	41 817 526 404*	$1.1 \cdot 10^{-4}$
Q9BE41	MH						84.9	8.94	223 519	$4.0 \cdot 10^{-5}$
A0JNJ5	MLa						7.9	0.83	20 801	$4.0 \cdot 10^{-5}$
Q0P571	MLr hemoglobin						7.2	0.76 4.3 [26]	18 882 31 007	$4.0 \cdot 10^{-5}$
P01966	α						48.5	2.09	15 053	$1.4 \cdot 10^{-4}$
P02070	β						51.5	2.21	15 954	$1.4 \cdot 10^{-4}$
	Milk casein	200 ml								
P02662	α-S1							2.00 [27]	22 974	$8.7 \cdot 10^{-5}$
P02663	α-S2							0.52 [27]	24 348	$2.1 \cdot 10^{-5}$
P02666	β							1.86 [27]	23 582	$7.9 \cdot 10^{-5}$
P02668	κ							0.66 [27]	19 104	$3.5 \cdot 10^{-5}$

Note: All molecular weights are calculated without posttranslational modifications.

* For myosin containing two subunits of MH, MLa, and MLr [14].

Then it was used for calculations.

In addition, it was assumed that probabilities of cleavage of peptide bonds of human and porcine pepsin are equal, as it was shown that their hydrolytic activity in relation to food proteins is almost the same [30].

RESULTS

Step-by-step comparison revealed in the EROP-Moscow database many oligopeptides, amino acid sequences of which were completely identical to fragments of proteins in the study. They contain two or more amino acid residues and demonstrate a diverse range of functional activity. Angiotensin I-converting enzyme inhibitor was the most abundant. Neuropeptides, hormones, immunoregulators, antimicrobial, and other regulatory oligopeptides were also found among them. However, quantitative estimates of activity for many regulatory oligopeptides are not available, so the fragments identical to these were excluded from the study.

We noted some repeated amino acid sequences of the fragments (up to 35). This repeatability is illustrated with the fragmentomes [6, 16] shown in Fig. 1. These data indicate that the number of different small fragments of actin and myosin is less than the number of theoretically possible fragments of a given length. Maximum length of identical fragments for actin is four amino acid residues, and 14 for three subunits of myosin. Similar fragmentomes were obtained for casein.

The portion of fragments non-cleavable by pepsin among all the formed ones was much smaller. Table 2 shows the data for all of these fragments for three pH values. The same table shows the values of EC_{50} and IC_{50} for oligopeptides identical to them, in increasing order for each protein. The results indicate 22 cases of fragment formation for all selected proteins except casein α-S2. Two fragments were formed at pH 1.0, nine fragments are generated at pH 2.5, and the largest number of fragments was obtained at pH 4.0 (18 fragments). Some fragments are formed both at pH 4.0 and 2.5, but no fragment can be formed at all three pH values. The longest fragment was that of β-hemoglobin, consisting of seven amino acid residues.

Nineteen fragments had primary structures identical to the amino acid sequence of oligopeptides with known angiotensin I-converting enzyme inhibitor activity EC_{50}. The **VK** fragment was encountered most often (seven times); it is formed from molecules of actin, myosin MLa (two times) and MLr, the β-chain of hemoglobin (two times), and casein β. The pair of surrounding amino acid residues in the precursor molecule was different in all these cases, and the formation was observed only at pH 4.0. The kinetic curves of accumulation of this fragment (Fig. 2a) show that the increase in the concentration of each fragment occurs slowly, almost linearly, and EC_{50} is achieved after 20 h or more.

Another enzyme-inhibiting fragment of **AF** was encountered four times – twice in myosin MLa and once in β-chain of hemoglobin and casein β. The kinetic curves in Fig. 2b show that the accumulation of this fragment, formed from part of the β-chain of hemoglobin 12-13, and the attainment of EC_{50} value also occurs within 20 h (pH 4.0) and more (pH 2.5). When formed from other proteins, these fragments are produced even more slowly.

More rapid accumulation occurs in the case of the angiotensin I-converting enzyme inhibitor fragment of casein α-S1, **AW**, time of attainment of EC_{50} of which at pH 4.0 is <6 h (Fig. 2c). At pH 2.5 the formation of this

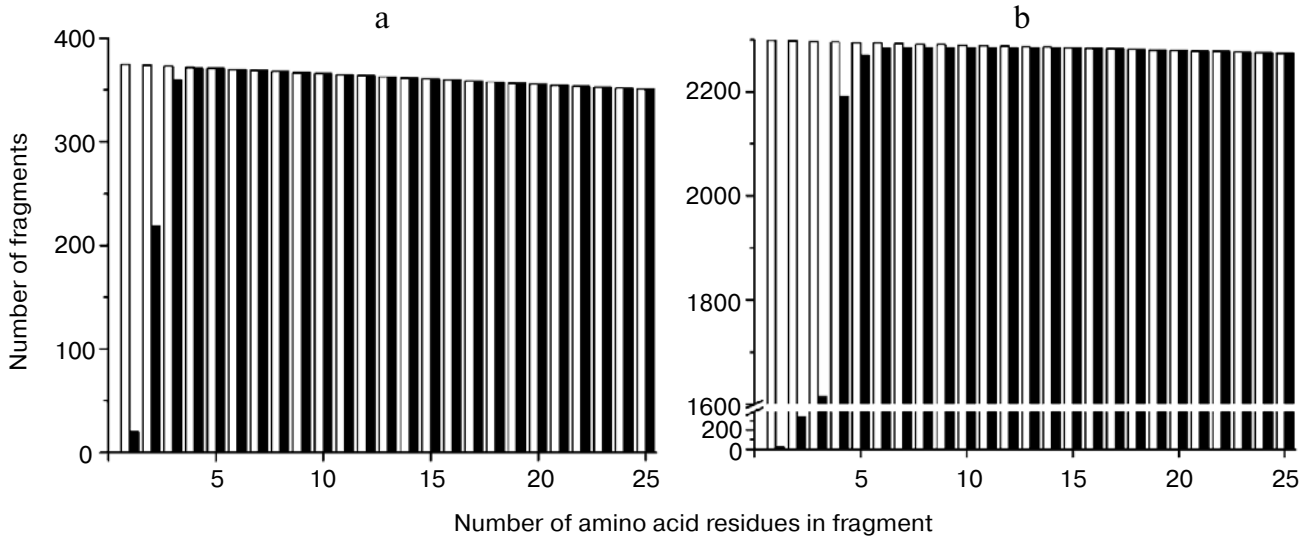

Fig. 1. Fragmentomes of actomyosin complex proteins. a) Actin; b) myosin (all three subunits). White columns, total number of theoretically possible fragments; black columns, number of different fragments.

Table 2. Probabilities ($\times 10^3$) of peptide bond cleavage by pepsin at three pH values in fragments of bovine actin, myosin, and casein

Fragment	pH 1.0								pH 2.5								pH 4.0								EC_{50}
	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	
Fragments of actin																									
KIWHHTF	1.75	0	0	1.63	0	2.25			**1.80**	**0**	**0**	**0**	**0**	**5.54**			0	0	0	0	0	2.97			5.1 [31]
LVKA	4.63	2.88	1.13						6.18	1.03	1.80						**5.10**	**0**	**1.06**						13 [32]
Fragment of myosin heavy chain MH																									
SIYK	**1.63**	**0**	**5.38**						0	0	4.25						**2.34**	**0**	**3.61**						3.7 [31]
Fragments of myosin light (alkaline) chain MLa																									
EVKK	1.38	2.88	1.00						2.57	1.03	1.93						**2.76**	**0**	**1.70**						13 [32]
FVKH	7.13	2.88	4.13						10.68	1.03	2.19						**7.01**	**0**	**7.01**						13 [32]
EAFL	4.13	1.38	5.00						**3.48**	**0**	**5.66**						**4.25**	**0**	**9.34**						15.2 [33]
EAFV	4.13	1.38	7.13						**3.48**	**0**	**10.68**						**4.25**	**0**	**7.01**						15.2 [33]
Fragments of myosin light (regulatory) chain MLr																									
NVKN	1.13	2.88	0						2.83	1.03	0						**4.67**	**0**	**5.31**						13 [32]
Fragments of hemoglobin α-chain																									
KYR	1.00	3.13							**2.19**	**0**							7.01	5.31							0.69 [34]*
AAWG	3.88	4.13	3.63						**3.09**	**0**	**5.15**						**4.25**	**0**	**4.25**						12 [35]
VNFK	4.13	7.88	4.13						0	9.14	489						**2.76**	**0**	**2.76**						16.3 [36]
Fragments of hemoglobin β-chain																									
VYPWTQRFF	5.00	0	0	0	1.25	0	0	2.50	1.29	2.57	9.65	0	1.29	0	0	8.63	**2.12**	**0**	**0**	**0**	**0**	**0**	**0**	**7.01**	2.9 [37]*
KVKV	2.63	2.88	2.63						1.80	1.03	1.80						**2.97**	**0**	**2.97**						13 [32]
KVKA	2.63	2.88	1.13						1.80	1.03	1.80						**2.97**	**0**	**1.06**						13 [32]
TAFW	2.50	1.38	8.38						**3.99**	**0**	**12.87**						**1.70**	**0**	**21.24**						15.2 [33]
VYPWTQ	**5.00**	**0**	**0**	**0**	**1.25**				1.29	2.57	9.65	0	1.29				2.12	0	0	0	0				45.2 [38]*
Fragment of casein α-S1																									
GAWY	2.50	4.13	4.13						**2.32**	**0**	**6.44**						**7.64**	**0**	**10.62**						12 [35]
Fragments of casein β																									
VVPPF	1.75	2.13	0	4.13					4.63	4.25	3.22	4.25					**1.91**	**0**	**0**	**7.01**					9 [39]
KVKE	2.63	2.88	1.88						1.80	1.03	0.64						**2.97**	**0**	**1.06**						13 [32]
QAFL	2.50	1.38	5.00						**5.54**	**0**	**5.66**						**2.97**	**0**	**9.34**						15.2 [33]
Fragments of casein κ																									
AIPPK	4.13	0	0	0					4.25	0	3.22	1.29					**4.67**	**0**	**0**	**2.12**					5 [39]
YPSYG	0	1.13	0	2.50					**2.57**	**0**	**0**	**2.83**					0	0	0	2.34					16 [32]

Note: Bold font indicates amino acid residues of a fragment, and regular font corresponds to residues surrounding it in the protein. Bold font also indicates probabilities for fragment excision (instead of cleavage).

* IC_{50}.

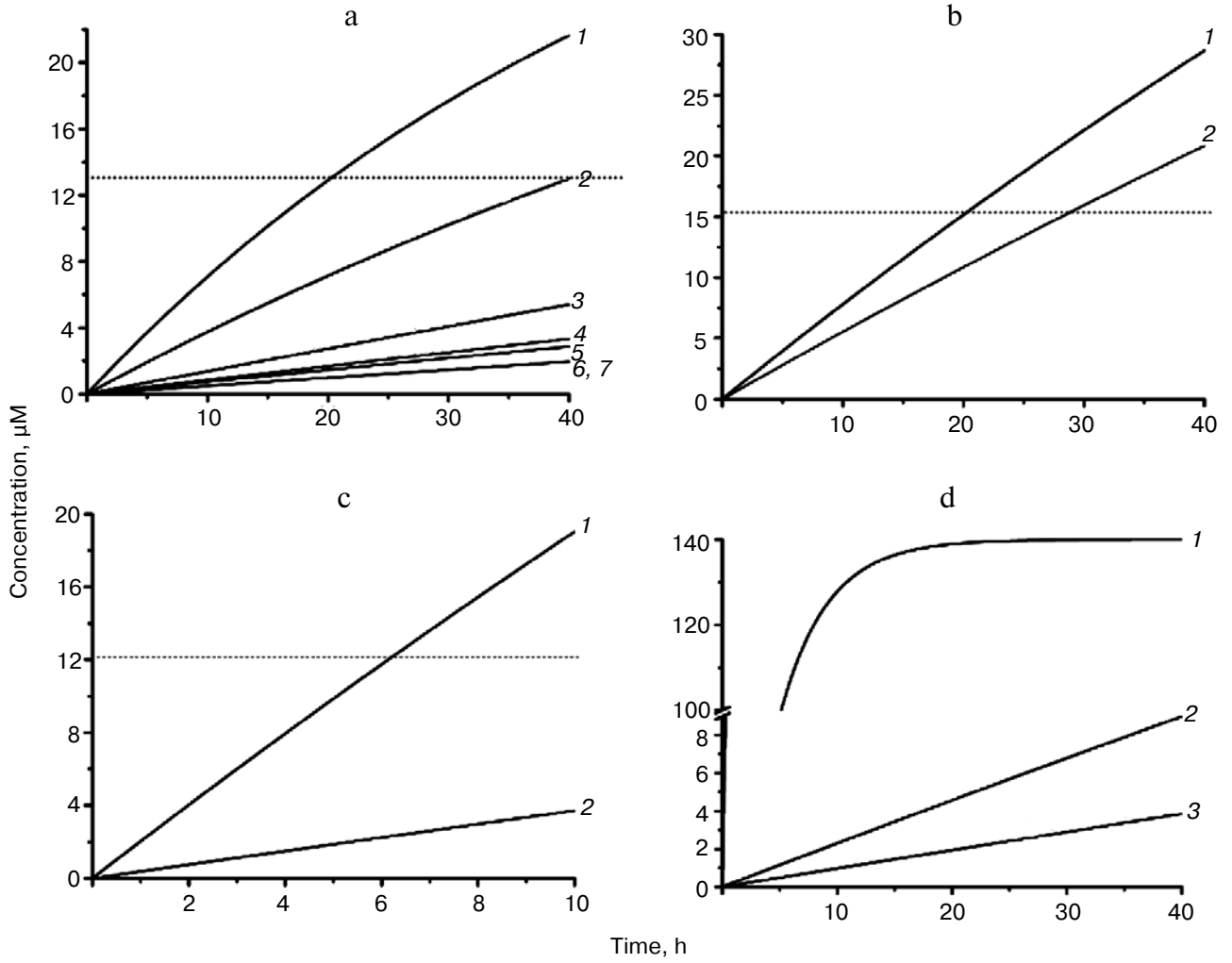

Fig. 2. Kinetics of fragments formation from food proteins. a) Angiotensin I-inhibiting dipeptide fragment **VK** formed from different parts of two separate proteins (pH 4.0, EC_{50} = 13 μM). Lines: *1-5*) myosin MLa 185-186 F**VK**H, myosin MLr 62-63 N**VK**N, hemoglobin β 17-18 K**VK**N, actin 17-18 L**VK**A, and myosin MLa 88-89 E**VK**K, respectively; *6*, *7*) β-chains of hemoglobin 59-60 K**VK**A and casein β 98-99 K**VK**E, respectively. b) Angiotensin I-converting enzyme inhibitor dipeptide fragment of β-chain of hemoglobin 12-13 T**AF**W, at pH 2.5 and 4.0 (lines *1* and *2*, respectively) and EC_{50} = 15.2 μM. c) Angiotensin I-converting enzyme inhibitor dipeptide fragment of casein α-S1 G**AW**Y at pH 4.0 and 2.5 (lines *1* and *2*, respectively) and EC_{50} = 12 μM. d) Angiotensin I-converting enzyme inhibitor dipeptide fragment of hemoglobin α 140-141 K**YR**, hemoglobin β 34-39 V**YWTQRF**F, and hemoglobin β 34-37 V**YPWT**Q (at pH 2.5, 4.0, 1.0, lines *1-3*, respectively). Amino acid residues of a fragment are shown in bold, surrounding residues are in normal font. Horizontal dashed lines in Fig. 2 (a-c) correspond to EC_{50}. IC_{50} values for Fig. 2d are shown in Table 2.

fragment is considerably slower. In the case of formation of this fragment from other proteins, the time needed to reach EC_{50} value is significantly longer (not shown in the figure).

Three fragments turned out to be well-known opioids – kyotorphin **YR** (a fragment of the hemoglobin α-chain [40]), hemorphin-4 **YPWT** [38], and hemorphin-7 **YPWTQRF** [37] (both are fragments of hemoglobin β-chain), IC_{50} values of which are known. All are formed at different pH values (Fig. 2d). Kyotorphin is a *C*-terminal fragment of hemoglobin α-chain, and its formation requires only one peptide bond A_{1-2} cleavage (A_3 and A_4 are absent). Therefore, the formation is very fast and reaches IC_{50} = 0.69 μM after 1 min. Hemorphin-7 is also formed relatively quickly – IC_{50} = 2.9 μM is attained in ~12 h. However, the concentration of less active hemorphin-4 (IC_{50} = 45.2 μM) after 40 h was only 3.8 μM.

Similar results, not represented in the illustrations, were obtained at different pH values for the remaining six fragments, which are also inhibitors of angiotensin I-converting enzyme inhibitor: α-chain of hemoglobin 97-98 (**NF**), myosin MH 352-353 (**IY**), casein β 84-86 (**VPP**), casein κ 36-38 (**PSY**), casein κ 108-110 (**IPP**), and actin 85-89 (**IWHHT**). For most of them the time of attainment of the corresponding values of EC_{50} (Table 2) is >40 h. Only the myosin MH 352-353 (**IY**) fragment has this time of 12.5 h.

DISCUSSION

The data suggest that fragments of food proteins can exist for a long time without being cleaved by pepsin. This time depends on the probabilities of cleavage of amino acid residues adjacent to the terminal amino acid residues of the fragment and on pH value. Obviously, the results can be valid only in the case of protein breakdown by enzymes in solution. This case corresponds to the data obtained for milk casein (Fig. 2, a and b). However, in the case of actin, myosin, and hemoglobin, not all molecules are available to pepsin molecules even on fractional consumption of food, i.e. real formed concentrations will be less than calculated. Data on homogenized meat may be closest to the actual results. This condition is satisfied in the case of homogenates used, for example, for the preparation of infant food or food for astronauts. Conformational changes of protein substrate may be an additional effect influencing the cleavage of food proteins by pepsin. For example, casein molecules change their conformation after thermal denaturation and coagulate when placed in an acid environment [41], and as a result their availability to enzyme molecules changes. These and other factors may either slow or speed the process of pepsin cleavage of the chosen proteins.

At the same time, the process of digestion increases the availability of substrate molecules to the enzyme molecules, which increases the concentration of the fragment. It should also be noted that not all possible dipeptides (400), tripeptides (8000), etc. were tested on whether they have a particular activity. For example, enzyme-inhibiting activity of oligopeptides is proven only for 61 dipeptides and 93 tripeptides. Therefore, it is clear that there may be other active fragments of examined food proteins that contribute to the pool of regulatory oligopeptides. This way, a number of processes promotes or prevents the accumulation of fragments formed from proteins.

The discovery of enzyme inhibitors (that affect not only angiotensin I-converting enzyme inhibitor) among the studied fragments suggests that the process of food proteins decomposition in the gastrointestinal tract may be regulated through proteolysis products. Neuropeptides that have opioid activity can also participate in the regulation of gastrointestinal tract smooth muscle contraction. In addition, the newly formed fragments, which have antimicrobial properties, can exert an effect directly on microbiota, complementing the immune defense of epithelial cells [42]. However, quantitative data on the effective concentrations of these fragments (as well as hormones, immunomodulators, and other oligopeptide regulators we found) are absent.

The residence time of beef meat in the stomach is about 3.5 h [43] (according to some sources, 6-8 h), while in most cases the time to reach an effective concentration of the fragment amounts to many hours. The exception is kyotorphin (Fig. 2d). However, chyme passes through the intestines much more slowly: it stays >3 h in the small intestines and from 30 to 70 h in the human large intestine [44]. It is therefore necessary to consider the possibility of fragments existing under the action of other enzymes secreted by the pancreas into the intestine, where the pH is alkaline for healthy persons [45].

Fragments that come from the stomach into the intestine mix there with endopeptidases (trypsin, chymotrypsin, and elastase), as well as exopeptidases (aminopeptidase and carboxypeptidase). All of these, except for pepsin, are active at higher pH values (Table 3). A detailed study on the probabilities of different peptide bond cleavages (as in the case of pepsin) was not conducted. However, it is known that trypsin cleaves a bond after arginine and lysine residues. Chymotrypsin predominantly cleaves bonds after tryptophan, tyrosine, and phenylalanine residues [18], and elastase cleaves bonds after residues with small side chains [55]. Carboxypeptidase cleaves aromatic nonpolar residues and residues of basic amino acids from the *C*-terminus, and aminopeptidases, respectively, cleave residues from the *N*-terminus [18].

Table 3. Range of acceptable pH values and maximal turnover numbers of pepsin and some human pancreas enzymes

Enzyme	EC	pH		Turnover number
		range	optimum	
Pepsin A	3.4.23.1	1.0-6.0 [46]	2.0 [46]	0.5 [23]
Trypsin	3.4.21.4	6.0->10.0 [47]	8.2-8.4 [47]	88.2 [48]
Chymotrypsin C	3.4.21.2	6.5-9.0 [47]	7.5 [47]	16.1 [49]
Elastase	3.4.21.36	7.3-9.2 [50]	8.3-8.8 [50]	0.11 [51]
Carboxypeptidase B	3.4.17.2	6.0-10.0 [52]	7.0 [52]	61 [53]
Aminopeptidase O	3.4.11.-		7.5 [54]	0.0004 [54]

Note: Only one turnover number is given for each pancreas enzyme. These data are slightly different in different publications. They depend on experimental conditions, in particular on the protein used as a substrate, but in general these values are comparable in order of magnitude.

Based on these data, we conclude that a number of protein fragments are formed even quicker in the intestine under the action of trypsin and chymotrypsin, the turnover number of which is greater than that of pepsin. Thus, **VK** fragments, not formed after pepsin action in the stomach, have their formation finished in the intestine, as they contain a lysine residue that is recognized by trypsin. Formation of kyotorphin **YR** also continues in the intestine, as in the original protein this fragment is preceded by a lysine residue. However, this neuropeptide, as well as the other two (**YPWT** and **YPWTQRF**), is cleaved by trypsin (**R**–**F** bond) and chymotrypsin (**W**–**T** bond). Therefore, a significant accumulation of these neuropeptides is only possible in the stomach. Chymotrypsin also cleaves the enzyme, which inhibits the **IWHHT** fragment containing aromatic amino acid residues (**W**–**H** and, possibly, **H**–**H** bonds). All other resulting fragments are not cleaved by trypsin and chymotrypsin or are cleaved very slowly (tested in PeptideCutter program: http://web.expasy.org/peptide_cutter).

Elastases can cleave bonds between small hydrophobic amino acid residues, for example, in the fragment of α-chain of hemoglobin A**AW**G (A–**A** bond). As a result, the formation of dipeptide **AW** continues, and its pool is replenished. However, the turnover numbers of elastases are small (less than or comparable to those of pepsin): for human pancreatic elastase-1 it is 0.55 sec^{-1}, and for elastase-2 it is 0.11 sec^{-1} [51]. Consequently, they exert relatively little influence on these fragments formed in the stomach. Under the actions of elastase, formation goes on for one fragment of **VK** (due to the additional L–**V** bond cleavage), **VPP** (cleavage of V–**V** bond), and **IPP** (A–**I**).

Carboxypeptidase also cleaves **VK** and **YR** fragments, which have a basic residue on the *C*-terminus, and their content in the intestines decreases rapidly due to the rather large turnover number of the enzyme. In the case of pancreatic aminopeptidase O, characterized by very low turnover number 0.0004 sec^{-1} [54], the arginine residue can be cleaved from the *N*-terminus, but we have not found any fragments with such residues. However, it should be noted that the available data on the specificity of the endo- and exopeptidases is insufficient to accurately assess their activity. In most cases, their concentrations in the gastrointestinal tract are unknown. Direct studies have only determined the content of trypsin in the human stomach, which is almost 20 times less than the pepsin content [29]. In addition, besides degradation under the action of the studied enzymes, skeletal muscles can go through autolysis [56], and numerous other human enzymes, proteases or peptidases, produced by the microflora of the gastrointestinal tract may affect them as well as milk proteins [57].

Regulatory oligopeptides that are fragments of food proteins act not only in the gastrointestinal tract. They can enter blood and lymph due to absorption. This absorption is caused by active transport, and in many cases the systems of oligopeptide transport work faster than systems of amino acid transport. Thus, the rate of transport of some dipeptides exceeds the rate of transport of the amino acids that form them [18]. Among the investigated oligopeptides this property is possessed by the **LY** oligopeptide [58], which is represented by two fragments in actin and four fragments in MH myosin. This dipeptide was given to rats one time *per os*, and after that the time was measured when it could be detected in blood plasma. This time was 30 min, and maximum concentration was achieved after 1.5 h. Approximately the same time was required for appearance in human blood plasma of angiotensin-I-converting enzyme inhibitor **VY** (represented in MH myosin twice), the action of which caused a decrease in blood pressure [59]. This confirms the possibility of expression of food protein fragment activity not only in the gastrointestinal tract.

Despite a number of obvious assumptions about the interaction of enzyme with substrate and lifetime of noncleavable fragments, we can assume that a dynamic pool of exogenous regulatory oligopeptides is formed in the body as a result of fragmentation of food proteins, and their functions are altered with formation of smaller and smaller fragments. These exogenous fragments in low concentrations (including less than EC_{50}) can correct regulatory processes carried out by endogenous oligopeptides. The discovery of the existence of such exogenous pool expands the significance and content of the hypothesis of a functionally continuous population (continuum) of endogenous natural oligopeptides [4]. Nevertheless, given the large number of uncertainties in the physical and chemical processes of food protein digestion, it is obvious that actual lifetimes and concentrations of the fragments can only be determined in direct experiments.

In future, acquisition of new data about structure and functions of natural oligopeptides will allow fuller characterization of functional resources of numerous but not yet studied protein fragments, to approach understanding of their role in evolution and to use this data in practice. For example, taking into consideration functional properties of food protein fragments can allow nutritionists to predict which food is preferable for a consumer, regarding individual acidity of gastrointestinal tract, and pharmacologists – to predict which peptide fragments would be reasonable to use as drugs or food additives.

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REFERENCES

1. Sewald, N., and Jakubke, H. D. (2002) *Peptides: Chemistry and Biology*, Wiley-VCH Verlag GmbH, Weinheim.

2. Zamyatnin, A. A., Borchikov, A. S., Vladimirov, M. G., and Voronina, O. L. (2005) *Neurochemistry (Moscow)*, **22**, 17-32.
3. Zamyatnin, A. A., Borchikov, A. S., Vladimirov, M. G., and Voronina, O. L. (2006) *Nucleic Acids Res.*, **34**, D261-D266.
4. Ashmarin, I. P., and Obukhova, M. F. (1986) *Biokhimiya*, **51**, 531-544.
5. Zamyatin, A. A. (2004) *Biochemistry (Moscow)*, **69**, 1276-1282.
6. Zamyatin, A. A. (2009) *Adv. Biol. Chem. (Moscow)*, **49**, 405-428.
7. Takagi, H., Shiomi, H., Ueda, H., and Amano, H. (1979) *Nature*, **282**, 410-412.
8. Takagi, H., Shiomi, H., Fukui, K., Hayashi, K., Kiso, Y., and Kitagawa, K. (1982) *Life Sci.*, **31**, 1733-1736.
9. Barkhudaryan, N., Kellerman, I., Lottspeich, F., and Galoyan, A. A. (1991) *Neurochemistry (Moscow)*, **10**, 146-154.
10. Ivanov, V. T., Karelin, A. A., Mikhaleva, I. I., Vaskovsky, B. V., Sviryaev, V. I., and Nazimov, I. V. (1992) *Russ. J. Bioorg. Chem.*, **18**, 1271-1311.
11. Benson, B., and Ebels, L. (1993) *Life Sci.*, **54**, PL437-PL443.
12. Ekeke, N. U., Shaw, C., Johnston, C. F., and Thim, L. (1992) *Regul. Peptides*, **40**, 140-140.
13. Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T. (1995) *J. Dairy Sci.*, **78**, 1253-1257.
14. Lehninger, A. (1982) *Principles of Biochemistry*, Worth Publishers, N. Y.
15. Zamyatin, A. A. (2007) *Neurochemistry (Moscow)*, **24**, 21-29.
16. Zamyatin, A. A. (2008) *Biophysics (Moscow)*, **53**, 329-335.
17. Zamyatin, A. A. (2009) *Biochemistry (Moscow)*, **74**, 201-208.
18. Schmidt, R. F., and Thews, G. (eds.) (1989) *The Human Physiology*, Springer-Verlag, Berlin-Heidelberg-New York.
19. Wu, C. H., Apweiler, R., Bairoch, A., Natale, D. A., Barker, W. C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M. J., Mazumder, R., O'Donovan, C., Redaschi, N., and Suzek, B. (2006) *Nucleic Acids Res.*, **34**, D187-D191.
20. Jones, R. G., and Landon, J. (2002) *J. Immunol. Methods*, **263**, 57-74.
21. Brier, S., Maria, G., Carginale, V., Capasso, A., Wu, Y., Taylor, R. M., Borotto, N. B., Capasso, C., and Engen, J. R. (2007) *FEBS J.*, **274**, 6152-6166.
22. Palashoff, M. H. (2008*) Determining the Specificity of Pepsin for Proteolytic Digestion*, *Chemistry Master's Theses*, Paper 1, http://hdl.handle.net/2047/d10016636.
23. Stenesh, J. (1998) *Biochemistry*, Plenum Press, N. Y., p. 89.
24. Radcliffe, D. V. (1998) *Stomach*, in *Compton's Encyclopedia on Line*, The Learning Company, Vol. 3.
25. Gnedenko, B. V., and Khinchin, A. Ya. (1964) *Elementary Introduction into Probabilities Theory* [in Russian], Fizmatgiz, Moscow, pp. 32-33.
26. Belitz, H.-D., Grosch, W., and Schieberle, P. (2008) *Food Chemistry*, Springer Verlag, Berlin-Heidelberg, p. 571.
27. O'Brien, P. J., Shen, H., McCutcheon, L. J., O'Grady, M., Byrne, P. J., Ferguson, H. W., Mirsalimi, M. S., Julian, R. J., Sargeant, J. M., Tremblay, R. R. M., and Blackwell, T. E. (1992) *Mol. Cell Biochem.*, **112**, 45-52.
28. Raikos, V. (2010) *Food Hydrocolloids*, **24**, 259-265.
29. Metheny, N. A., Stewart, B. J., Smith, L., Yan, H., Diebold, M., and Clouse, R. E. (1997) *J. Parenter. Enteral Nutr.*, **21**, 279-285.
30. Chiang, L., Sanchez-Chiang, L., Mills, J. N., and Tang, J. (1967) *J. Biol. Chem.*, **242**, 3098-3102.
31. Yokoyama, K., Chiba, H., and Yoshikawa, M. (1992) *Biosci. Biotechnol. Biochem.*, **56**, 1541-1645.
32. Li, C.-H., Matsui, T., Matsumoto, K., Yamasaki, R., and Kawasaki, T. (2002) *J. Peptide Sci.*, **8**, 267-274.
33. Matsui, T., Li, C.-H., and Osajima, Y. (1999) *J. Peptide Sci.*, **5**, 289-297.
34. Kiso, Y., Kitagawa, K., Kawai, N., Akita, T., Takagi, H., Amano, H., and Fukui, K. (1983) *FEBS Lett.*, **155**, 281-284.
35. Minghetti, P. P., Ruffner, D. E., Kuang, W. J., Dennison, O. E., Hawkins, J. W., Beattie, W. G., and Dugaiczyk, A. (2000) *Biol. Pharmacol. Bull.*, **23**, 879-883.
36. Suetsuna, K. (1998) *J. Nutr. Biochem.*, **9**, 415-419.
37. Zhao, O., Garreau, L., Sannler, F., and Piot, J. M. (1997) *Biopolymers. Peptide Sci.*, **43**, 75-98.
38. Brantl, V., Gramsch, C., Lottspeich, F., Henschen, A., Jaeger, K. H., and Herz, A. (1985) *Eur. J. Pharmacol.*, **111**, 293-294.
39. Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T. (1995) *J. Dairy Sci.*, **78**, 1253-1257.
40. Takagi, H., Shiomi, H., Ueda, H., and Amano, H. (1979) *Eur. J. Pharmacol.*, **55**, 109-111.
41. Fox, P. F., and McSweeney, P. L. H. (1998) *Dairy Chemistry and Biochemistry*, Kluwer Academic/Plenum Publisher, N. Y., p. 152.
42. Zamyatin, A. A., and Voronina, O. L. (1998) *Adv. Biol. Chem. (Moscow)*, **38**, 165-197.
43. Smith, C. A., Fishback, H. R., Bergeim, O., Rehfuss, M. E., and Hawk, P. B. (1919) *Am. J. Physiol.*, **49**, 204-221.
44. Degen, L. P., and Fillips, S. F. (1996) *Gut*, **39**, 299-305.
45. Fallingborg, J. (1999) *Dan. Med. Bull.*, **46**, 183-196.
46. Piper, D. W., and Fenton, B. H. (1965) *Gut*, **6**, 506-508.
47. Goldberg, D. M., Campbell, R., and Roy, A. D. (1969) *Gut*, **10**, 477-483.
48. Gombos, L., Kardos, J., Patthy, A., Medveczky, P., Szilagyi, L., Malnasi-Csimadia, A., and Graf, L. (2008) *Biochemistry*, **47**, 1675-1684.
49. Bence, M., and Sahin-Toth, M. (2011) *FEBS J.*, **278**, 4338-4350.
50. Fujimoto, K., Ogawa, M., Saito, N., Kosaki, G., Minamiura, N., and Yamamoto, T. (1980) *Biochim. Biophys. Acta*, **612**, 262-267.
51. Largman, G., Brodrick, J. W., and Geokas, M. C. (1976) *Biochemistry*, **15**, 2491-2500.
52. Marinkovic, D. V., Marinkovic, J. N., Erds, E. G., and Robinson, C. J. G. (1977) *Biochem. J.*, **163**, 253-260.
53. Edge, M., Forder, C., Hennam, J., Lee, I., Tonge, D., Hardern, I., Fitton, J., Eckersley, K., East, S., Shufflebotham, A., Blakey, D., and Slater, A. (1988) *Protein Eng.*, **11**, 1229-1234.
54. Diaz-Perales, A., Quesada, V., Sanchez, L. M., Ugalde, A. P., Suarez, M. F., Fey, A., and Lopez-Otin, C. (2005) *J. Biol. Chem.*, **280**, 14310-14317.
55. Del Mar, E. G., Largman, C., Brodrick, J. W., Fassett, M., and Geokas, M. C. (1980) *Biochemistry*, **19**, 468-472.
56. Ilian, M. A., Bekhit, Ael-D., and Bickerstaffe, R. (2004) *Meat Sci.*, **66**, 387-397.
57. Paulo, J. A., Lee, L. S., Wu, B., Banks, P. A., Steen, H., and Conwell, D. L. (2010) *Proteomics Clin. Appl.*, **4**, 715-725.
58. Nakashima, E. M., Kudo, A., Iwaihara, Y., Tanaka, M., Matsumoto, K., and Matsui, T. (2011) *Anal. Biochem.*, **414**, 109-116.
59. Matsui, T., Tamaya, K., Seki, E., Osajima, K., Matsumo, K., and Kawasaki, T. (2002) *Biol. Pharm. Bull.*, **25**, 1228-1230.