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Bioactive peptides from beef products fermented by acid whey – *in vitro* and *in silico* study

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ABSTRACT: This research investigated the potential of beef products with acid whey to release bioactive peptides and thereby emphasize their health-promoting potential. Peptide sequences were isolated and identified by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Firstly, the antihealth properties (toxicity, allergenicity) of the peptides were estimated based on the peptide sequences. Next, their health-promoting potential was demonstrated based on an *in silico* analysis by determining their bioactivity scores (PeptideRanker). Their various biological actions were also determined using BIOPEP-UWM tools. We presented peptide sequences with properties relevant to ensuring good health and well-being, including cardiovascular system, nervous and immune systems, or their support for the maintenance of general homeostasis. We obtained information on generation of biologically active peptides in uncured beef with acid whey and it can be considered as a new knowledge as it contributes to science development of functional and nutraceutical foods. In the long term, this information can be used in designing products with desired nutritional and health-promoting properties that are important for the well-being and for preventing the occurrence of noncommunicable diseases.

Keywords: biological active peptides, allergenity, uncured beef, healthy meat product

Introduction

Action mechanisms of biologically active peptides derived from meat and meat products are described in the literature (Ha and Zemel, 2003; Lafarga and Hayes, 2014). However, the search for new food sources of biologically active peptides is the current trend in food science. Fermented beef marinated in acid whey could be such kind of product. The available literature provides a detailed account on the biological activity of specific whey components, including whey proteins and peptides as well as amino acids (Tavares and Malcata, 2013). Most experiments confirming bioactivity of whey proteins were conducted *in vitro* and *in vivo* (Pihlanto-Leppälä, 2000; Lasik et al., 2011; Zhou et al., 2017; Le Maux et al., 2018). Biologically active substances with high health potential in acid whey include immunoglobulin A, β-lactoglobulin, α-lactoglobulin, glutamine, lactoferrin, lactoferricine, lisozyme, and glutathione. A previous study reported that the use of acid whey as replacement of sodium nitrite in the manufacturing of meat products inhibits the growth of undesirable microflora, creates a pink-color and has significant impact on the sensory profile of products acceptable by consumers (Wójciak and Dolatowski, 2015; Wójciak et al., 2015; Wójciak and Solska, 2016). During meat fermentation, acid whey contributes not only to meat tenderness, but also to generation of peptide fractions in products, which may cause significant physiological effect in human body, such as antioxidant and blood pressure lowering effects, at least *in vitro* (unpublished data). Interaction of meat proteolytic factors with those delivered with whey can result in the formation of specific sequences. The delivery of biologically active ingredients, such as peptides, can modulate or activate endogenous regulatory mechanisms in the human body thereby limiting the occurrence of lifestyle diseases (Garcia et al., 2017; Martínez-Sánchez et al., 2017; Montoro-García et al., 2017). Therefore, this research was conducted to investigate the capabilities of beef products with acid whey to release the bioactive peptides after ripening and one month of storage at 4 °C and thereby to emphasize the health-promoting potential of these meat products.

Materials and Methods

Sample preparation

Semimembranosus muscle (*m. semimembranosus*) from Limousine cattle (live weight around 400-450 kg, organic breeding system) were used in this study. After 48 h of slaughter, semimembranosus muscles were marinated in acid whey for 24 h at 4 °C. Each variant (about 6 kg beef per variant) was immersed in 1.5 L of acid whey. Then, the meat was salted with sea salt at 3 % on the meat weight and placed to rest for 24 h at 4 °C. After salting, 1 % of glucose was added to the meat. Products were subjected to 31 d of ripening at 16 °C and relative humidity 75-85 %. After ripening, the beef was cold-smoked for 1 h at 26 °C with oak-alder wood chips and then vacuum packaged and stored at 4 °C. Products were tested after one month of cold storage. The experiments were performed in triplicate.

Extraction and identification of peptides

Peptides were isolated according to Mora et al. (2010). After concentration in the evaporator, peptides were dissolved in 2 mL of 0.01 M HCL and subjected to further chromatographic analysis. The peptide mix-

ture was separated using nanoACQUITY (Waters) liquid chromatography instruments and Orbitrap Velos Mass Spectrometer. The peptide mixture was applied to a RP-18 column using a gradient of acetonitrile (0-35 % AcN) for over 180 min, in the presence of 0.05 % formic acid at a flow rate of 250 nl min^{-1}. The data was processed by Mascot Distiller then Mascot Search and later compared to the Uniprot database. The search parameters for precursor ions and mass tolerance products were 10 ppm and 0.1. Da. The study was repeated three times.

Anti-health properties

The toxic and allergenic properties of peptide sequences were determined using an *in silico* method. The peptide sequences were assessed for potential toxicity using ToxinPred web server (Gupta et al., 2013). The prediction method was based on the support vector machine (SVM) and the SVM threshold value of 0.0 was applied for toxicity prediction (Lafarga et al., 2015; Kęska and Stadnik, 2016a). The above-mentioned peptides were also assessed for potential allergenic properties using tools available at the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz et al., 2008). A profile for epitopes was created, which shows the potential presence and location of epitope fragments in a protein sequence. The frequency of the occurrence of allergenic fragments in a protein sequence (*A* parameter) was also determined according to the equation: $A = a\ N^{-1}$; where a is the number of fragments defined as epitope and N is the number of amino acid residues of protein. Parameter *A* determined for epitopes was marked as A_{EP} in this study for a better interpretation of results.

Health-promoting properties

The chromatographic analysis was used to assess potential of health-promoting properties of ripening beef meat products. The overall bioactivity of the obtained peptide sequences was determined using the tools available on the server Peptide Ranker (http://bioware.ucd.ie /~compass/biowareweb/Server_pages/peptideranker.php). The profile of potential biological activity of peptides and frequency of bioactive fragments occurrence in a protein sequence (parameter *A*) was determined using the tools available at the BIOPEP-UWM database. Parameter *A* is available in tab "Calculations" and is estimated according to a procedure similar to described previously (i.e. $A = a\ N^{-1}$), wherein a is the number of fragments with a given activity in a protein sequence.

Results and Discussion

Profiling of ripening and refrigeration storage products

A typical LC-MS/MS base peak chromatogram because of chromatographic analyses was shown in Figure 1. After three independent repeats, 1,464 peptides were identified. Among these peptides, 452 peptide sequences, whose presence was confirmed in each repetition, were selected for further analysis. The potential bioactivity index was determined for each identified peptide, based on bioinformatic tools (PeptideRanker). PeptideRanker is a server for predicting bioactive peptides based on the novel N-to-1 neural network, which allows an assessment to confirm if the examined sequence is biologically active. The numerical indicator ranged from 0 (no activity) to 1 (maximum bioactivity). As an example, Table 1 provides 62 (14 %) peptides for which the value of the bioactivity index was greater than 0.5.

Selected peptides had from 7 to 21 amino acids in the sequence and showed bioactivity from 0.5 to 0.97, according to the PeptideRanker score. Of these, the highest potential activity was determined for the octapeptide FPMNPPKF and the nonapeptide FPM-

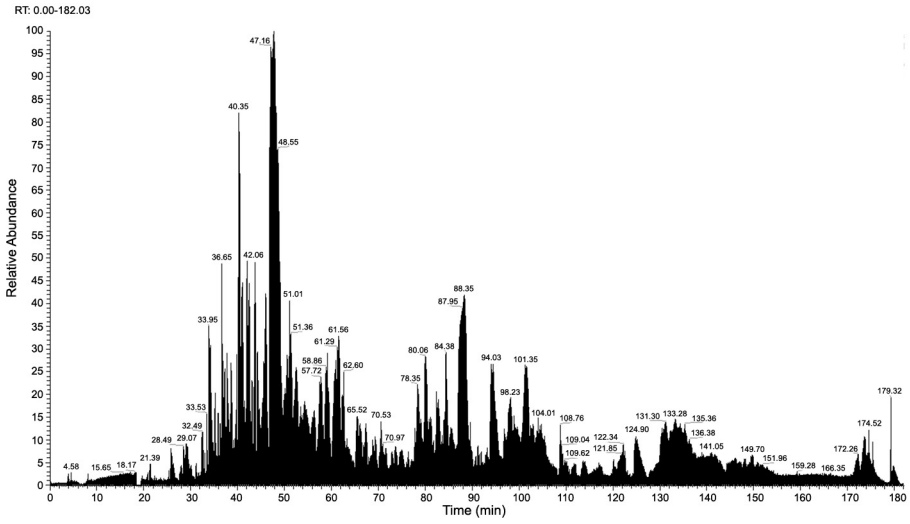

Figure 1 – Representative base peak chromatogram spectra ethanol-soluble fragments from ripening beef with acid whey.

Table 1 – Peptides with potential high biological activity - an in silico study.

No	Peptide	Mass [Da]	Peptide ranker	No	Peptide	Mass [Da]	Peptide ranker
1	VPPLPLI	747.48	0.73	32	DQVFPMNPPKFD	1433.66	0.59
2	VPPLPLL	747.48	0.84	33	GGPAPEAITDKIF	1442.74	0.63
3	TPIPWLS	812.44	0.67	34	PVVPPLIPPKIPEG	1451.87	0.63
4	LKLAGFGL	817.51	0.55	35	APPIQSPLPVIPHQ	1492.84	0.53
5	DRHGGFKP	914.46	0.60	36	GSGLVKAGFAGDDAPR	1516.76	0.58
6	FPMNPPKF	976.48	0.97	37	DDHFLFDKPVSPL	1528.76	0.75
7	HAKHPSDFG	994.46	0.51	38	DDHFLFDKPVSPI	1528.76	0.60
8	VPIPTMPIR	1022.59	0.68	39	MPKFDLGPLLSEPL	1555.83	0.68
9	GNPELILPVP	1047.60	0.55	40	FPMNPPKFDKIED	1576.76	0.55
10	SDGTLLQPLK	1070.60	0.58	41	GSGLVKAGFAGDDAPRA	1587.80	0.61
11	GEAAPYLRKS	1090.58	0.50	42	FAGDDAPRAVFPSIVG	1617.82	0.64
12	FPMNPPKFD	1091.51	0.87	43	DFGADAQAAMSKALEL	1636.78	0.52
13	PVVVPPFLQP	1091.63	0.56	44	IDDHFLFDKPVSPI	1641.84	0.55
14	AGNPELILPVP	1118.63	0.55	45	IDDHFLFDKPVSPL	1641.84	0.71
15	VGVNGFGRIGR	1130.63	0.53	46	PTIPEEEAKKLFPKG	1682.92	0.53
16	TAPKGKVGGRW	1155.65	0.57	47	LIDDHFLFDKPVSPI	1754.92	0.50
17	AAQYKVLGFHG	1189.62	0.54	48	LIDDHFLFDKPVSPL	1754.92	0.65
18	AGNPELILPVPA	1189.67	0.51	49	GRPTPKSSWEFDGKAK	1789.91	0.58
19	GTAPKGKVGGRW	1212.67	0.70	50	GHPETLEKFDKFKHL	1824.95	0.63
20	GLSDGEWQIVL	1215.61	0.51	51	GAPSFPLGSPLSSPVFPRAG	1940.02	0.89
21	GLSDGEWQLVL	1215.61	0.60	52	STGAAKAVGKVIPELNGKLT	1953.13	0.56
22	MSADAMLKALLG	1219.63	0.54	53	HPSDFGADAQAAMSKALEL	1957.92	0.59
23	DAGELDFSGLLK	1263.63	0.67	54	SQPDVDGFLVGGASLKPEF	1961. 97	0.85
24	PEPAKSAPAPKKG	1276.71	0.54	55	AIPEGQFIDSKKASEKLL	1973.08	0.60
25	AGTAPKGKVGGRW	1283.71	0.73	56	STGAAKAVGKVIPELNGKLTG	2010.15	0.67
26	TAPKGKVGGRWK	1283.75	0.52	57	ASHLPSDFTPAVHASLDKF	2039.01	0.56
27	DQVFPMNPPKF	1318.64	0.83	58	LDDLPGALSELSDLHAHKL	2043.06	0.55
28	AVGKVIPELNGKL	1336.81	0.52	59	FTGHPETLEKFDKFKHL	2073.07	0.50
29	GTAPKGKVGGRWK	1340.77	0.64	60	ASHLPSDFTPAVHASLDKFL	2152.09	0.68
30	IDDHFLFDKPV	1344.67	0.62	61	FTGHPETLEKFDKFKHLK	2201.16	0.50
31	APPIQSPLPVIPH	1364.78	0.61	62	EQQQLIDDHFLFDKPVSPL	2268.14	0.52

NPPKFD (bioactivity score 0.97 and 0.87, respectively). These sequences were assigned to the myosin chain, as a source of origin (Uniprot ID: Q9BE40 and Q9BE41). The above-mentioned peptides were characterized by a particularly high potential activity for inhibiting the action of enzymes: angiotensin-converting enzyme ACE-I inhibitor (A = 0.8750 and A = 0.7778, respectively) and dipeptidyl peptidase IV (DPP IV) inhibitor (A = 0.8750 and A = 0.7778, respectively). In turn, the GAPSFPLGSPLSSPVFPRAG bioactivity (PeptideRanker score = 0.89), a peptide derived from desmin (Uniprot ID: O62654) showed potential bioactivity as ACE-I and DPP IV inhibitor (A = 0.7000 for both). Triosephosphate isomerase (Uniprot ID: Q5E956) was also a source of high biological activity (PeptideRanker score = 0.85) as ACE-I inhibitor (A = 0.5789) and DPP IV inhibitor (A = 0.4211).

Bioactive peptides derived from food may be multifunctional and exhibit two or more different biological activities. The active dipeptides or tripeptides, such as enzyme inhibitors or antioxidants, showed the strongest impact on the physiological function in human body (Erdmann et al., 2008). The high bioactivity potential determined for the FPMNPPKF peptide is due to the presence of shorter fragments with high biological effects in its sequence (for example, shorter fragments in the peptide structure may act either as ACE-I inhibitors - FP, MNPPK, MNP, NPP, and PPK or DPP IV inhibitors - PP, FP, NP, KF, MN, PK, and PM).

In silico analysis of potential anti-health properties of the identified peptides

The potential of anti-health properties for each peptide sequence was evaluated, *inter alia* by their toxicity, based on an *in silico* approach. For this purpose, a tool available in ToxinPred webserver was used. This solution only allows the analysis of peptides that are not longer than 30 amino acids in length and therefore data on the possible toxic activities of the three identified peptides (ISDAIIHVLHAKHPSDFGADAQAAMSKALEL, IYKKLRDKETPSGFTLDDVIQTGVDNPGHPF, and EIYKKLRDKETPSGFTLDDVIQTGVDNPGHPF) are not available. The remaining sequences of the peptides did not show toxic properties.

Meat and meat products, which contribute to the nutritional and functional values of the daily human

diet, can also be the cause of diseases to populations, including allergies. Food allergies are the major public health problem worldwide. Doctors report that such incidences are increasing steadily, but so far, a universal method to fight them has not been developed. Epidemiological data report that food allergies affect 8 % of children and 5 % of adults (van Hengel, 2007; Sicherer and Sampson, 2014). Milk proteins are some of the most dangerous food allergens. The use of acid whey in preparation of ripening beef is likely to affect allergenic properties of the product. Therefore, in order to assess whether peptides released from ripening beef can be used as functional food ingredients for human consumption, their allergenicity was determined. The potential of peptides to induce food allergies was evaluated, based on the information in the BIOPEP-UWM database available at "Allergenic proteins and their epitopes". Within the sequences of analyzed peptides, epitopes (i.e., a part of a macromolecule recognized by the immune system), responsible for causing the allergic response of organisms, were located. The search for local identity sequence using epitopes as a sequence of queries is the simplest possible strategy for bioinformatics to find new allergens. Among all peptides, ten sequences (2 %) have potential allergenic properties (Table 2).

All potentially allergenic peptide sequences had myoglobin as the source of origin (Figure 2). These results correspond with reports of other authors (Fuentes et al., 2004; Fiocchi et al., 2005).

Table 2 – Profile of epitope occurrence in peptide sequences of ripening beef meat product[1].

No	Peptide sequences	Location	A_{EP} parameter for epitope
1	IRLFTGHPET**LEKFDK**	11-16	0.0182
2	TGHPET**LEKFDK**FK	7-12	0.0625
3	TGHPET**LEKFDKF**KH	7-12	0.0588
4	FTGHPET**LEKFDK**FK	8-13	0.0667
5	GHPET**LEKFDK**FKHL	6-11	0.0667
6	TGHPET**LEKFDK**FKHL	7-12	0.0556
7	FTGHPET**LEKFDK**FKH	8-13	0.0625
8	FTGHPET**LEKFDK**FKHL	8-13	0.0526
9	FTGHPET**LEKFDK**FKHLK	8-13	0.0500
10	IRLFTGHPET**LEKFDK**FKHL	11-16	0.0417

[1]All sequences were designated as the fragments of bovine myoglobin (Uniprot ID: P02192).

10	20	30	40	50
MGLSDGEWQL	VLNAWGKVEA	DVAGHGQEVL	**IRLFTGHPET**	**LEKFDKFKHL**
60	70	80	90	100
KTEAEMKASE	DLKKHGNTVL	TALGGILKKK	GHHEAEVKHL	AESHANKHKI
110	120	130	140	150
PVKYLEFISD	AIIHVLHAKH	PSDFGADAQA GFHG	AMSKALELFR	NDMAAQYKVL

Figure 2 – Bovine myoglobin sequences (Uniprot ID:P02192). Identified fragments with epitopes are listed in bold. The potentially allergenic sequence is underlined.

The occurrence frequency of epitopes in the protein sequence (indicated as A_{EP} parameter) was determined. A_{EP} was understood as the ratio of the number of fragments defined as epitopes to the number of amino acid residues in the protein sequence. For the identification of proteins as allergens that are able to cross-react with previously known allergens, the WHO recommends the following official bioinformatics criteria: presence of a common fragment containing at least 6-8 amino acid residues or of a fragment containing at least 80 amino acid residues with an identity of at least 35 % (Goodman, 2006; Ivanciuc et al., 2009). All sequences obtained have this fact in common, the six amino acid domain LEKFDK, residues identical to the corresponding fragment of a query peptide sequence, according to the official WHO criteria. It is a linear epitope of the allergen Bos d 5, characteristic of the allergenic bovine (*Bos taurus*) protein beta-lactoglobulin, gen. var. A (Dziuba et al., 2013; Restani et al., 2009). Beta-lactoglobulins - Bos d 5 are thermostable food allergens, which are resistant to proteolytic enzymes and hydrochloric acid. This feature does not allow the processes and action of digestive enzymes to change their allergenicity. All sequences containing epitope LEKFDK are assigned as myoglobin fragment based on processing the Mascot Distiller followed by Mascot Search and in comparison to the Uniprot database. In order to determine whether the application of acid whey can affect the allergenic properties of beef products, the sequences of myoglobin (Uniprot ID: P02192) and beta-lactoglobulin (Uniprot ID: P02754) were compared using the basic local alignment search tool (BLAST; Figure 3) (Goodman et al., 2016). Regions with similar sequence of proteins were not found, except for a single significant adjustment of short segment, which turned out to be the epitope LEKFDK. Thus, identified sequences from bovine meat tissue and the use of acid whey for beef production cannot additionally strengthen allergenicity of the final product. However, *in silico* methods use a variety of physicochemical properties (mainly amino acids search) of proteins that can be utilized; however, a strong correlation between the structural characteristics and the operation of sensitization has not been confirmed. Using innovative methods for *in silico* prediction of allergenicity will largely depend on the choice of databases and algorithms to be developed, standardized, and, most importantly, empirically validated (Hayes et al., 2015). Determining the potential allergen is not enough to prove allergenicity of peptides. Further studies based on biochemical and biological tests are needed to confirm the allergy potential.

Experimentally, it was found that the linear sequences of the epitopes in bovine whey proteins are also present in milk of other animals such as goats (*Capra hircus*), sheep (*Ovis aries*), and buffalo (*Bubalus bubalis*) (Table 3). Thus, proteins from milk of these species should be classified as allergens, based on the local sequence identity, which exhibit cross-reactivity with bovine milk protein (Minkiewicz et al., 2011; Restani et

Table 3 – Epitope structure LEKFDK present in various food proteins[1].

ID of allergenic proteins[2]	Name of allergenic protein
14	beta-lactoglobulin, gen. var. A, precursor, bovine (*Bos taurus*), Allergen Bos d 5
77	beta-lactoglobulin, precursor, sheep (*Ovis aries*), allergen Ovi a BLG
100	beta-lactoglobulin, goat (*Capra hircus*), allergen Cap h BLG
108	beta-lactoglobulin, water buffalo (*Bubalus bubalis*), allergen Bub b BLG

[1]based on information available at "profiles of epitope occurrence in your sequence" offered at BIOPEP-UWM database; [2]ID in BIOPEP-UWM database.

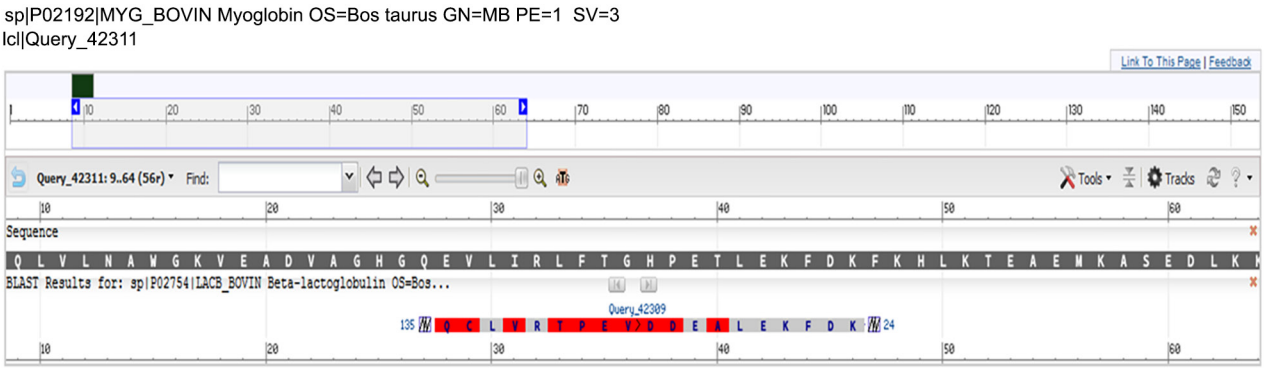

Figure 3 – Graphical results of alignment for myoglobin and beta-lactoglobulin from *Bos taurus* using Blast tool (identical fragment has been shaded in light gray).

al., 2009). Therefore, possibly, cross-reaction may occur even after the ingestion of ripening beef, due to the presence of myoglobin.

In silico analysis of potential nutritional properties of the identified peptides

Biologically active peptides derived from food sources are short sequences released by the breakdown of protein molecules that exert physiological, hormone-like effects on humans besides the contribution of their nutritional value. Integrity and activity of food ingredients change during food production, which generates various breakdown/transformation products under the influence of biochemical and enzymatic reactions. This also applies to the proteolytic transformation of proteins under different conditions with the influence of endogenous and exogenous environmental factors. Depending on the amino acid sequence, these peptides may exhibit a variety of activities. The ripening of beef products with acid whey after one month of refrigeration storage are a potential source of biopeptides, which shows 17 different activities: dipeptidyl peptidase IV inhibitor (DPP IV inhibitor; 452), angiotensin converting enzyme inhibitor (ACE-I inhibitor; 449), antioxidative (268), stimulating (203), the bacterial permease ligand (82) inhibitor (other than DPP IV inhibitor or ACE-I inhibitor, 82), antithrombotic (62), hypotensive (57) regulating (46) antiamnesic (45); immunomodulating (43) ubiquitin-mediated (28), opioid (16) neuropeptide (6), anticancer (2), anorectic (2) and chemotactic (2) (numbers in parentheses denote the number of peptides with the biological activity). We present peptide sequences (maximum ten) with properties relevant to the proper maintenance of good health and well-being - including cardiovascular effects by regulating the blood pressure or oxidation of stress (Table 4) effects of nervous and immune systems (Table 5), or supporting maintenance of general homeostasis (Table 6).

Regarding the daily diet, the modulation of immune response can occur in the gut associated lymphoid tissue (GALT), which is located within the gastrointestinal track. It plays a very important role, because most antigens enter the human body through the intestinal mucosa. Efficient functioning of the immune system at this level prevents the intestinal barrier and penetration of pathogens into the body (Kuśmierska and Fol, 2014). Therefore, food is a potential source of immunomodulatory compounds that can be used to control immune responses (Santiago-Lopez et al., 2016). The action mechanism of bioactive peptides for controlling and preventing diseases consists of suppressing or stimulating some immune responses. Bovine milk, eggs, mushroom, soybean, and wheat as the source of immunoactive peptides from different food materials were presented in the literature (Hartmann and Meisel, 2007; Agyei and Danquah, 2012). However, to the best of knowledge, information about fermented/ripening meat products as a source of factors affecting the immune system is limited. Kęska and Stadnik (2016b) demonstrated the prospective of pork protein as a potential source of immunomodulators in *in silico* approach. Moreover, these studies enriched by the results of chromatographic analyses provide insights into the sequence of peptides obtained by protein degradation *in vivo* in ripening beef product with acid whey, which enable a better assessment of the

Table 4 – Peptide sequences influencing the cardiovascular system.

Activity	Sequences	Protein	*A*
DPP IV inhibitor	VPPLPLI[1]	NI[2]	1.1429
	VPPLPLL[1]	NI	1.1429
	VPTVPLP	NI	1.0000
	QETVAPGATVGQVLGA	NI	0.9375
	PVVPPLIPPKIPEG	Q8MKH6(*Troponin T, slow skeletal muscle*)	0.9286
	APPIQSPLPVIPH	NI	0.9231
	TEGGATLTVK	Q9BE41 (*Myosin-2*)	0.9000
	PVVVPPFLQPE	P02666 (*Beta-casein*)	0.9091
	DQVFPMNPPKF	Q9BE40 (*Myosin-I*)	0.9091
	APPPPAEVPEVHEEVHE	Q8MKI3 (*Troponin T, fast skeletal muscle*)	0.8824
ACE-I inhibitor	LKLAGFGL	NI	0.8750
	FPMNPPKF	Q9BE40 (*Myosin-I*)	0.8750
	GSGLVKAGFAGDDAPRAVFPS	Q3ZC07 (*Actin, alpha cardiac muscle 1*); P68138 (*Actin, alpha skeletal muscle*)	0.8571
	AGTAPKGKVGGRW	Q8MKI3 (*Troponin T, fast skeletal muscle*)	0.8426
	GSGLVKAGFAGDDAPR	P68138 (*Actin, alpha skeletal muscle*)	0.8235
	EKAGAHLKGGAKR	NI	0.8125
	GSGLVKAGFAGDDAPRAVFPSIVG	Q3ZC07 (*Actin, alpha cardiac muscle 1*); P68138 (*Actin, alpha skeletal muscle*)	0.7919
	PVVPPLIPPKIPEG	Q8MKH6 (*Troponin T, slow skeletal muscle*)	0.7857
	FPMNPPKFD	Q9BE40 (*Myosin-I*)	0.7778
	EKAGAHLKG	P10096(*Glyceraldehyde-3-phosphate dehydrogenase*)	0.7778
Antioxidant	IGAEVYHHLK	Q3ZC09 (*Beta-enolase*)	0.5000
	IGAEVYHHLKG	Q3ZC09 (*Beta-enolase*)	0.4545
	EKAGAHLKG	P10096 (*Glyceraldehyde-3-phosphate dehydrogenase*)	0.4444
	MRIGAEVYHHLK	Q3ZC09 (*Beta-enolase*)	0.4167
	IGAEVYHH	Q3ZC09 (*Beta-enolase*)	0.3750
	EKAGAHLKGGAK	P10096 (*Glyceraldehyde-3-phosphate dehydrogenase*)	0.3330
	MEKAGAHLKGGAK	P10096 (*Glyceraldehyde-3-phosphate ehydrogenase*)	0.3077
	MRIGAEVYHH	Q3ZC09 (*Beta-enolase*)	0.3000
	KKGHHEA	P02192 (*Myoglobin*)	0.2857
	TPIPWLS	Myozenin-1 (Q8SQ24)	0.2857
Antitrombotic	NEEIDEMLKEAPGPINF	Q3SZE5 (*Myosin regulatory light chain 2, ventricular/cardiac muscle isoform*)	0.1765
	VGPEVEK	Q3T0P6 (*Phosphoglycerate kinase 1*)	0.1429
	FPMNPPKF	Q9BE41 (*Myosin-2*)	0.1250
	FPMNPPKFD	Q9BE41 (*Myosin-2*)	0.1110
	DQVFPMNPPKF	Q9BE41 (*Myosin-2*)	0.0909
	GGPEAGKSEQPEN	Q3T149 (*Heat shock protein beta-1*)	0.0769
	EDQVFPMNPPKFD	Q9BE41 (*Myosin-2*)	0.0769
	TPIPWLSSGEPVD	Q8SQ24 (*Myozenin-1*)	0.0769
	PVVPPLIPPKIPEG	Q8MKH6 *(Troponin T, slow skeletal muscle)*	0.0714
	DVIQTGVDNPGHPF	Q9XSC6 (*Creatine kinase M-type*)	0.0714
Hypotensive	EKFDKFKH	P02192 (*Myoglobin*)	0.2500
	IRLFTGHPETLEKFDKFKHL	P02192 (*Myoglobin*)	0.1500
	TGHPETLEKFDKFK	P02192 (*Myoglobin*)	0.1429
	FTGHPETLEKFDKF	P02192 (*Myoglobin*)	0.1429
	GHPETLEKFDKFKH	P02192 (*Myoglobin*)	0.1429
	TGHPETLEKFDKFKH	P02192 (*Myoglobin*)	0.1333
	FTGHPETLEKFDKFK	P02192 (*Myoglobin*)	0.1333
	GHPETLEKFDKFKHL	P02192 (*Myoglobin*)	0.1333
	FTGHPETLEKFDKFKH	P02192 (*Myoglobin*)	0.1250
	FPMNPPKF	Q9BE41 (*Myosin-2*)	0.1250

[1]VPPLPLI and VPPLPLL also show activity as ACE-I inhibitor (*A* = 0.8571); inhibitor (*A* = 0.1429). In order to avoid an excessive number of repetitions they were omitted from the table; [2]NI = not identified.

Table 5 – Peptide sequences influencing the immune and nervous systems.

Activity	Sequences	Protein	*A*
Immunomodulating	LKTEAEMK	P02192 (*Myoglobin*)	0.1250
	KKKGHHEAE	P02192 (*Myoglobin*)	0.1111
	TEAEMKASEDLK	P02192 (*Myoglobin*)	0.0833
	GGILKKKGHHEAE	P02192 (*Myoglobin*)	0.0769
	KTEAEMKASEDLK	P02192 (*Myoglobin*)	0.0769
	TEAEMKASEDLKK	P02192 (*Myoglobin*)	0.0769
	LKTEAEMKASEDLK	P02192 (*Myoglobin*)	0.0714
	KTEAEMKASEDLKK	P02192 (*Myoglobin*)	0.0714
	FDKFKHLKTEAEMK	P02192 (*Myoglobin*)	0.0714
	LKGVIKAKYGKDA	Q3ZC09 *(Beta-enolase)*	0.0769
Opioid	YYPLKSMTEQEQQLIDDHF	Q9XSC6 *(Creatine kinase M-type)*	0.0500
	GAPSFPLGSPLSSPVFPRAG	O62654 (*Desmin*)	0.0500
	GKYYPLKSMTEQEQQLIDDH	Q9XSC6 *(Creatine kinase M-type)*	0.0476
	GKYYPLKSMTEQEQQLIDDHF	Q9XSC6 *(Creatine kinase M-type)*	0.0455
	GKYYPLKSMTEQEQQLIDDHFL	Q9XSC6 *(Creatine kinase M-type)*	0.0435
	FKGKYYPLKSMTEQEQQLIDDH	Q9XSC6 *(Creatine kinase M-type)*	0.0435
	KGKYYPLKSMTEQEQQLIDDHFL	Q9XSC6 *(Creatine kinase M-type*	0.417
	GEFKGKYYPLKSMTEQEQQLIDDH	Q9XSC6 *(Creatine kinase M-type)*	0.0400
	FKGKYYPLKSMTEQEQQLIDDHFL	Q9XSC6 *(Creatine kinase M-type)*	0.0400
	TGEFKGKYYPLKSMTEQEQQLIDDH	Q9XSC6 *(Creatine kinase M-type)*	0.0385
Neuropeptide	GEAAPYLRKS	Q9BE39 (*Myosin-7*); Q9BE40 (*Myosin-1*); Q9BE41 (*Myosin-2*)	0.1000
	AAPYLRKSEK	Q9BE39 (*Myosin-7*); Q9BE40 (*Myosin-1*); Q9BE41 (*Myosin-2*)	0.1000
	SRYLGKGVLK	Q3ZC09 (*Beta-enolase)*	0.1000
	RPRHQGVMVGMGQKD	P60712 (*Actin*)	0.0667
	VADKAAYLQGLNSADLLK	Q9BE40 *(Myosin-1)*	0.0556
	LAESHANKHKIPVKYLEF	P02192 (*Myoglobin*)	0.0556
Regulating and antiamnestic[2]	NEEIDEMIKEAPGPINF	NI[1]	0.1765
	NEEIDEMLKEAPGPINF	Q3SZE5 *(Myosin regulatory light chain 2, ventricular/cardiac muscle isoform)*	0.1765
	VGPEVEK	Q3T0P6 *(Phosphoglycerate kinase1)*	0.1429
	GVDNPGHP	Q9XSC6 *(Creatine kinase M-type)*	0.1250
	GGPEAGKSEQPEN	Q3T149 (*Heat shock protein beta-1*)	0.0769
	GGPAPEAITDKIFQ	Q08DP0 (*Phosphoglucomutase-1*)	0.0714
	DVIQTGVDNPGHPF	(*Creatine kinase M-type*)	0.0714
	QETVAPGATVGQVLGA	NI	0.0625
	MPKFDLGPLLSEPL	Q8SQ24 (*Myozenin-1*)	0.0714
	TKQEYDEAGPSIVHR	P68138 (*Actin, alpha skeletal muscle*)	0.0667

[1]NI = not identified; [2]VPTVPLP was determined as strong antiamnestic peptide (*A* = 0.1429).

biological potential of bovine proteins. The results of the analyses are shown in Table 5. The role of myoglobin as a good source of immunomodulatory peptides was also emphasized.

Opioid peptides act as opioid-like hormones by interacting with specific receptors in the nervous, endocrine, and immune systems (Martínez-Alvarez, 2013). Opioid peptides are small molecules, which are synthesized *in vivo* and may function as hormones and neurotransmitters. Typical opioid peptides are endorphin, enkephalin, and prodynorphin that can be produced by the human body. As noted by Lafarga and Hayes (2014), most studies on the production of meat-based opioid peptides are based on blood hydrolysates (hemorphins). However, there are no reports on the generation of opioid peptides from other food-based meat-origin proteins. Thus, the results presented in this study contribute to this knowledge by addressing the missing gap in this area and highlighting the role of creatine kinase M-type as the best source of opioid peptides (Table 5).

Conclusion

The peptidomics and bioinformatics approaches used in this study indicated the peptide sequences obtained from beef product with acid whey have high potential for modulating various functions of the human system, especially as an ACE-I inhibitor and DPP IV inhibitor or as an antioxidant agent. In addition, all peptide fragments exhibited more than one biological activity due to the presence of shorter fragments in the sequences. These fragments are likely to be released

Table 6 – Peptide sequences with other activities.

Activity	Sequences	Protein	A
Stimulating	KKEEEELVALKERIEK	Q8MKI3 (*Troponin T, fast skeletal muscle*)	0.3750
	ISDAIIHVL	P02192 (*Myoglobin-2*)	0.2220
	AEEEYPDLSKHNNH	Q9XSC6 (*Creatine kinase M-type*)	0.2143
	EVHTKIISE	Q9BE40 (*Myosin-1*)	0.2000
	ISDAIIHVLH	P02192 (*Myoglobin*)	0.2000
	HIITHGEEKD	Q3SZE5 (*Myosin regulatory light chain 2, ventricular/cardiac muscle isoform*)	0.2000
	REVHTKIISE	Q9BE40 (*Myosin-1*)	0.2000
	GNPELILPVP	Q3ZC09 (*Beta-enolase*)	0.2000
	PTIPEEEAKKLFPKG	O77834 (*Peroxiredoxin-6*)	0.2000
	KAEEEYPDLSKHNNH	Q9XSC6 (*Creatine kinase M-type*)	0.2000
Bacterial permease ligand	KKGHHEA	P02192 (*Myoglobin*)	0.4429
	KKKGHHE	P02192 (*Myoglobin*)	0.4286
	KKKGHHEA	P02192 (*Myoglobin*)	0.3750
	KKKGHHEAE	P02192 (*Myoglobin*)	0.3333
	NILKKKGHHE	NI[1]	0.3000
	GGILKKKGHHE	P02192 (*Myoglobin*)	0.2727
	NILKKKGHHEA	NI	0.2727
	NILKKKGHHEAE	NI	0.2500
	GGILKKKGHHEA	P02192 (*Myoglobin*)	0.2500
	GGILKKKGHHEAE	P02192 (*Myoglobin*)	0.2308
Inhibitor	EKFDKFKH	P02192 (*Myoglobin*)	0.2500
	GGILKKKGHHEA	P02192 (*Myoglobin*)	0.7500
	TGHPETLEKFDKFK	P02192 (*Myoglobin*)	0.1429
	FTGHPETLEKFDKF	P02192 (*Myoglobin*)	0.1429
	GHPETLEKFDKFKH	P02192 (*Myoglobin*)	0.1429
	TGHPETLEKFDKFKH	P02192 (*Myoglobin*)	0.1333
	FTGHPETLEKFDKFK	P02192 (*Myoglobin*)	0.1333
	GHPETLEKFDKFKHL	P02192 (*Myoglobin*)	0.1333
	IRLFTGHPETLEKFDKFKHL	P02192 (*Myoglobin*)	0.1500
	MVEMEKKLEKGQSIDDMIPAQK	Q9XSC6 (*Creatine kinase M-type*)	0.6364
Activing ubiquitin-mediated	LKLAGFGL	NI	0.1250
	QEVQITLAARLG	NI	0.0883
	EITALAPSTMKIK	P60712 (*Actin*)	0.0769
	DLAGNPELILPVP	Q3ZC09 (*Beta-enolase*)	0.0769
	DLAGNPELILPVPA	Q3ZC09 (*Beta-enolase*)	0.0714
	LAESHANKHKIPVK	P02192 (*Myoglobin*)	0.0714
	FRAAVPSGASTGIYE	Q3ZC09 (*Beta-enolase*)	0.0667
	HLAESHANKHKIPVK	P02192 (*Myoglobin*)	0.0667
	FRAAVPSGASTGIYEA	Q3ZC09 (*Beta-enolase*)	0.0625
	FAGDDAPRAVFPSIVG	Q3ZC07 (*Actin, alpha cardiac muscle 1*); P68138 (*Actin, alpha skeletal muscle*)	0.0625
Anticancer	PVVVPPFLQP	P02666 (Beta-casein)	0.1000
	PVVVPPFLQPE	P02666 (Beta-casein)	0.0909
Anorectic and chemotactic	NEEIDEMIKEAPGPINF	NI	0.0588
	NEEIDEMLKEAPGPINF	Q3SZE5 (*Myosin regulatory light chain 2. ventricular/cardiac muscle isoform*)	0.0588

[1]NI = not identified.

during digestion and absorption in the human gastrointestinal tract as intact fragments (with preserved biological activity) reach a specific site of action in human body. Thus, consumption of dietetic biopeptides from uncured beef fermented with acid whey seems to provide further benefits to the health of humans against lifestyle diseases. Due to the natural protein origin as well as potential properties to enhance health, these products could be used as ingredients in functional foods or nutraceuticals. However, the implementation of effective and cost-effective production strategies for functional meat-based products on an industrial scale primarily requires standardization of analytical methods to determine the satisfactory health-promoting effects of the released peptides, evaluation of sensory properties for consumer acceptance, and, most importantly, well-planned clinical trials to provide

evidence to support health claims, which must be taken into account during the later stages of research on ripening beef with acid whey.

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Authors' Contributions

Conceptualization: Kęska, P.; Wójciak, K.M.; Stadnik, J. Data acquisition: Kęska, P.; Wójciak, K.M.; Stadnik, J. Data analysis: Kęska, P.; Wójciak, K.M.; Stadnik, J. Design of Methodology: Kęska, P.; Wójciak, K.M.; Stadnik, J. Writing and editing: Kęska, P.; Wójciak, K.M.; Stadnik, J.

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