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Animal and Plant Proteins as Precursors of Peptides with ACE Inhibitory Activity – An *in silico* Strategy of Protein Evaluation

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Summary

This paper presents a modern *in silico* approach useful in the evaluation of proteins as a source of ACE inhibitors. All protein sequences analyzed were derived from the BIOPEP database. To determine the protein value, the following criteria of evaluation were applied: the profile of potential biological (ACE inhibitory) activity of a protein, the frequency of the occurrence of fragments with ACE inhibitory activity (A) and the potential biological activity of a protein (B). The results, based on a statistical analysis, indicate that milk proteins can be a better source of ACE inhibitors than wheat gliadins. Moreover, all analyzed gliadins possessed more potent ACE inhibitors than chicken meat proteins. No significant differences were observed when comparing A values between soy globulins and β-lactoglobulins. Although criteria such as the profile of potential biological activity of protein, as well as parameters A and B, can be suitable tools in protein evaluation, the proteolytic digestion of protein needs to be considered. Moreover, computerised methods of classifying proteins according to different algorithms are often subjective due to discretion in interpretation of the results.

Key words: proteins, ACE inhibitors, BIOPEP database, *in silico* methods, biological activity, functional food

Introduction

There are many lifestyle diseases which cause public health problems worldwide (*1*). These diseases include cardiovascular problems (*e.g.* atherosclerosis, stroke or myocardial infarction) (*2*) as well as their major predisposing factors such as hypertension and diabetes (*3*). Overconsumption of cheap, energy-dense food and decreased physical activity have contributed to the growth of obesity in both Western and developing countries. Data from 2007 show that around 1.1 billion adults worldwide are overweight and 312 million of them are obese (*3*).

Apart from recommending a healthy lifestyle to prevent the diseases of civilization, many innovative nutritional strategies to reduce the main risk factors have been elaborated and their effects have been scientifically demonstrated (*4*). These strategies include dietary changes or the consumption of specifically targeted functional foods and supplements. For example, some food products and beverages have the appearance of 'normal food' but contain some specific components that can reduce blood pressure and cholesterol level (*4*). Food-derived bioactive peptides represent a source of health-enhancing components (*5*). Such peptides contribute to the physiological and sensory properties of protein-rich food. The health benefits of protein-derived peptides have been the subject of increasing commercial interest in health-promoting markets based on the novel concept of 'personalized nutrition' (*5*). Improvement of the biological functions of peptides may lead to providing new

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therapeutic applications for the prevention or treatment of chronic diseases (*6*).

Food products, especially dairy ones, are rich in ACE inhibitory (*i.e.* reducing blood pressure), antimicrobial, immunomodulating and many other biologically-active peptides (*5*). Bioactive peptides have also been found in meat, fish, seafood and eggs (*7*). Depending on the initial protein source, the enzyme applied and process conditions, the peptide biological activities can differ. For example, peptides released from soy protein showed anticarcinogenic, antioxidant and hypotensive activity (*6*).

Modern *in silico* techniques have become common in the area of food science. Techniques such as QSAR (quantitative structure-activity relationship) involving PCS (principal component similarity), HSA (homology similarity analysis) or RCO (random centroid optimization) have been applied successfully in designing peptides derived from food proteins (*8*). For example, homology similarity analysis (HSA) developed by Nakai *et al.* (*9*) facilitated the elucidation of the structure-function relationships of lactoferricin derivatives. The RCO procedure was applied for the modification of a single site of sixteen amino acids at the site of *Bacillus stearothermophilus* neutral protease to improve its thermostability (*10*). A number of peptides have been discovered, usually *in vitro*, based on their biological activities or *in silico*, based on their sequence similarities. Searching databases such as Swiss-Prot and Trembl and using BLAST alignment tools and other *in silico* methods, all currently known bioactive peptides and their precursor proteins can be extracted and provide information about the function and similarities between sequence motifs (*11–13*). Another bioactive peptide database, BIOPEP, provides data on over 2000 peptides with various activities (*14*). BIOPEP can be a useful tool in the area of prediction of the possibilities to release biologically active peptides (*15*) as well as the classification of proteins based on specially designed criteria (*16*).

According to Minkiewicz *et al.* (*17*) there is still a need to study and develop new technologies, both experimental and computational, which retain or even enhance the activity of bioactive peptides in food systems. Thus, in this study a method is proposed involving *in silico* techniques to compare plant and animal proteins as a source of ACE inhibitors which should be a suitable tool in the potential evaluation of protein sequences. Due to the fact that bioactive peptides are a well-known group occurring in their precursor sequences, it was assumed that peptides with the above-mentioned activity should appear in the great majority of proteins to be compared. This is consistent with previous studies which indicated that motifs with ACE inhibitory activity usually dominated in the analyzed protein sequences (*18*).

Materials and Methods

Protein sequences were extracted from the BIOPEP database available on the Internet at *http://www.uwm.edu.pl/biochemia* (*14,16*). The sequences analyzed in this study can be found in the BIOPEP database with the following ID numbers: 1076, 1077, 1078, 1079, 1081, 1082, 1083, 1085, 1086, 1087, 1088, 1089, 1090, 1091, 1092, 1093, 1094, 1095, 1097, 1098, 1099, 1100, 1101, 1102, 1103, 1104, 1105, 1106, 1107, 1108, 1109, 1111, 1112, 1113, 1114, 1115, 1116, 1117, 1120, 1121, 1122, 1126, 1128, 1130, 1133, 1134, 1140, 1141, 1142, 1143, 1144, 1148, 1149, 1150, 1152, 1153, 1154, 1155, 1161, 1162, 1163, 1164, 1167, 1168, 1169, 1170, 1171, 1172, 1173, 1174, 1175, 1176, 1177, 1178, 1179, 1180, 1181, 1182, 1183, 1184, 1186, 1193, 1196, 1197, 1198, 1199, 1200, 1273, 1317, 1318, 1319, 1320, 1365, 1550 and 1564.

The sequences of peptides with ACE inhibitory activity which are available in BIOPEP were derived from the literature resources. According to the references, these peptides are mainly described as ACE (angiotensin I-converting enzyme, EC 3.4.15.1) inhibitors. This means they inhibit the hydrolysis of angiotensin I to angiotensin II, which is known as one of the most important vasoconstrictors. Some of the ACE inhibitors showed an antihypertensive effect *in vivo* in spontaneously hypertensive rats (*19*).

BIOPEP is systematically and continuously updated with the 'new' sequences of peptides with different activities. It practically means that any single insertion of the 'new' peptide to the database or its deletion from it affects the values of discriminants A and B.

To compare the protein sequences, the following criteria were applied: (*i*) the profile of potential biological (ACE inhibitory) activity of a protein which is defined as the type and the location of ACE inhibitory fragment in a protein sequence; (*ii*) the occurrence frequency of the fragments with an ACE inhibitory activity in polypeptide chain (A) described by the equation given below:

$$A = \frac{a}{N} \qquad /1/$$

where a is the number of fragments with the ACE inhibitory activity, and N is the number of amino acid residues; (*iii*) potential biological (ACE inhibitory) activity of a protein (B in μM^{-1}):

$$B = \frac{\sum_{i=1}^{k} \frac{a_i}{EC_{50i}}}{N} \qquad /2/$$

where a_i is the number of repetitions of the i-th bioactive fragment in protein sequence, EC_{50i} is the concentration of the i-th bioactive peptide corresponding to half of its maximal activity (μM), k is the number of different fragments with ACE inhibitory activity, and N is the number of amino acid residues (*16*).

The above-mentioned discriminants of protein value were computed automatically by BIOPEP after the selection of the protein sequence to analyze (see a toolbar called 'Analysis' available in protein and bioactive peptide databases).

Due to the fact that BIOPEP is a universal database containing peptides with over forty types of activities, the EC_{50} (if available), which characterizes the given activity of the peptide, is taken into consideration to calculate B. According to the references concerning the ACE inhibitors, the measure of their activity is IC_{50}, defined as the sample concentration required to inhibit 50 % of the ACE activity (*20*). The ACE activity can be quantified colorimetrically, fluorometrically, radiochemically and chromatographically (*21*). The popular assays to de-

termine the ACE activity involve hippuryl-histidyl-leucine (HHL) (*22*) and/or furanacryloyl-L-phenylalanylglycylglycine (FAPGG) (*21*) as substrates. According to the HHL assay, the above-mentioned substrate is cleaved by angiotensin-converting enzyme (ACE) to release hippuric acid, which can be quantified spectrophotometrically at 228 nm. Thus, ACE inhibitory peptides will inhibit the formation of hippuric acid and it will affect the absorbance value (the lower the value, the more potent the peptide) (*22*). In the second assay, FAPGG is hydrolyzed to furanacryloyl-L-phenylalanine (FAP) and glycylglycine (GG), and the absorption spectrum is between 328 and 352 nm (*21*).

To conclude, if the BIOPEP user decides to calculate the potential ACE inhibitory activity for the given protein (B), the value of IC_{50} will be the component of the equation defined as parameter B.

A *t*-test was performed to find statistically significant differences between groups of proteins – precursors of ACE inhibitors. This test was performed using Statistica v. 8 software.

Results and Discussion

The profiles of the potential ACE inhibitory activity of the analyzed proteins revealed the presence of ACE inhibitors. The number of fragments with this activity that appeared in the protein sequences varied depending on the length of the protein chain (the longer the chain, the higher the probability to find more fragments in it) (*23*). As an example of such a profile, the ACE inhibitory characteristics of chicken connectin (*Gallus gallus*) are presented in Fig. 1. Fragments which are ACE inhibitors are underlined. The entire sequence of this protein consists of 1021 amino acid residues and includes 162 fragments with the above-mentioned activity. They are mostly di- and tripeptides. It should be noted that some of the biopeptides are part of the longer sequence, *e.g.* the chain position 519–527 (YKDGKKLKE) includes the following ACE inhibitory dipeptides: YK, DG, GK, KL and KE. This is important in terms of easier absorption of short peptides to blood in comparison with free amino acids (*23*).

The shortest protein analyzed was a fragment of monellin B (BIOPEP ID-1169), which consists of fifty amino acid residues. Twenty-one ACE inhibitory peptides (two tripeptides and nineteen dipeptides) were found in this protein fragment.

Milk is known as a complex of various nutrients and bioactive components (*24*). By submitting milk to an analysis of potential ACE inhibitory activity profiles for all analyzed sequences, it was confirmed that milk pro-

Chicken (*Gallus gallus*) connectin 1 – BIOPEP ID 1118

1. KS**RLRRRR**[1]ER**EITEI**TSEEEE**EIE**MVQHVHREFS**PP**S**RL**L**RRR**S**LSPT**YI**ELM**RP**VSEL**IRPR**SR**PP**EESE**RR**S**PT**PERT**RPR**SPSPVS**TE**RSLS**RF**ER

101. M**ARF**D**IF**S**RY**ESMKSALK**TQK**T**ME**R**KYEV**L**TQ**QPFTLDH**APR**ITLRMRSHR**VP**C**GH**NT**RF**I**LN**VQS**KPT**AD**VKW**YH**NGIELQ**ESSKIHFSNT**SGV**LTL**EI**

201. LDCHIDD**SGT**Y**RA**VCT**NYKGE**CSD**YA**TLDV**TGGD**YTTYSSQ**RR**DE**EVPRS**I**LP**DLTR**TEAYA**VS**SF**K**KA**T**AAEA**SSS**VREVKS**EV**SATRESLL**SY**EHHAS

301. SE**EK**ITASE**EK**SLEERTV**HKAF**KSTLPATILT**KPR**SITVS**EGE**T**ARF**SCDV**DGEPAPT**ITW**VRAGQ**PIVSS**RRF**QITR**TQ YK**STF**EI**SLVQ**IA**D**EGSY**T

401. V**VE**NS**EGR**Q**EAH**FTLTVQR**KRIPEKA**ITS**PPR**IKSPE**PRVK**SPEP**VK**SP**KRVK**SPEPISTPS**KA**KSP**PGD**K**TAPVEK**VQL**PT**AS**PPK**I**KE**Q**LKA**ET**LGD**K

501. V**KL**SCA**VE**SSVLS**IREVAWYKDGKKLKE**DHHF**KFHYAADGT**YELKIHNL**TE**SD**KGEY**TC**EIMGEGGI**SKT**NF**QF**TGQVFK**NIHSQVQSVSETPKS**VEKGD**

601. KV**LA**VS**TQK**KSS**AATEEKAAIEEV**IKKSIV**TE**D**VK**Q**LQ**AE**IRA**SS**TQ**MTVS**EGQK**VT**LKA**N**IPGA**S**EVKW**V**LNGME**LRNSDDY**RYGISGS**NHTLTIK**KA**S

701. **NK**D**EGI**LTC**EGK**TD**EGT**IKCQYV**LTF**S**KE**PSNEP**AF**I**TQ**PKSQNVN**EGQ**DV**LF**TC**EVSGD**PSP**EVEW**LRNNQP**IA**VSSHM **RA**TRSKNTYSL**EIR**N**AA**VSD

801. **TGKY**T**VKA**K**NYHGQ**CSATASLT**VFPLIE**E**PPKEV**VLKT**SGDA**SMHE**SF**SSQ**SF**QM**AA**SKQ**EASF**S**SF**SSSSM**TE**T**KF**ASM SAKSMSSM**KE SF**VEMSSSSI

901. **MGK**SSMAQLESSTSKMLK**SGVRGVPPKIEA**LPSDISID**EGK**VLTLSC**AFSGEPAPEI**TWYCR**GR**KITSQD Q**QGRFHIE**TS EDLTTLIIMDV**QK**N**DGGIY**T

1001. **LNLG**NE**FGT**D SATVNIN**IR**SI *Total number of ACE inhibitory fragments=162*

ACE inhibitory fragments: **ILP** [ID-2642][2], **RL** [ID-3257], **IR** [ID-3258], **RY** [ID-3380], **IY** [ID-3383], **VF** [ID-3384], **KW** [ID-3386], **RFH** [ID-3387], **TAP** [ID-3406], **RF** [ID-3489], **HY** [ID-3494], **FP** [ID-3502], **VPP** [ID-3524], **PR** [ID-3537], **LSP** [ID-3541], **IRA** [ID-3547], **LF** [ID-3551], **YG** [ID-3553], **AY** [ID-3563], **VFK** [ID-7488], **PL** [ID-7513], **AW** [ID-7543], **IRP** [ID-7547], **GEP** [ID-7554], **VK** [ID-7558], **IA** [ID-7562], **LKA** [ID-7569], **IP** [ID-7581], **RP** [ID-7582], **AF** [ID-7583], **AP** [ID-7584], **LA** [ID-7585], **KR** [ID-7586], **VP** [ID-7587], **RA** [ID-7588], **YA** [ID-7589], **AA** [ID-7590], **IF** [ID-7593], **GI** [ID-7596], **GM** [ID-7597], **GA** [ID-7598], **AG** [ID-7600], **GH** [ID-7601], **GR** [ID-7603], **KG** [ID-7604], **FG** [ID-7605], **DA** [ID-7606], **GS** [ID-7607], **GV** [ID-7608], **MG** [ID-7609], **GQ** [ID-7610], **GK** [ID-7611], **GT** [ID-7612], **HG** [ID-7614], **GE** [ID-7615], **GG** [ID-7616], **QG** [ID-7617], **SG** [ID-7618], **LG** [ID-7619], **GD** [ID-7620], **TG** [ID-7621], **EG** [ID-7622], **EA** [ID-7623], **NG** [ID-7624], **PG** [ID-7625], **VR** [ID-7628], **PAP** [ID-7633], **LTF** [ID-7638], **PPK** [ID-7645], **QK** [ID-7680], **DG** [ID-7681], **NY** [ID-7682], **NF** [ID-7683], **SY** [ID-7684], **SF** [ID-7685], **KY** [ID-7691], **KF** [ID-7692], **KL** [ID-7693], **YK** [ID-7697], **NK** [ID-7698], **RR** [ID-7741], **AR** [ID-7742], **KA** [ID-7743], **EY** [ID-7752], **KP** [ID-7810], **GEP** [ID-7817], **EI** [ID-7826], **IE** [ID-7827], **EV** [ID-7828], **VE** [ID-7829], **TE** [ID-7830], **LQ** [ID-7831], **LN** [ID-7832], **PT** [ID-7833], **TQ** [ID-7834], **AH** [ID-7835], **PP** [ID-7836], **EW** [ID-7838], **ME** [ID-7839], **EK** [ID-7840], **KE** [ID-7841], **HK** [ID-7844]

[1]some short chain sequences overlap, *e.g.* fragment RLRRRR includes the ACE inhibitors RL and RR. Thus the total number of peptides in the profile of connectin is 162

[2]ID number of the fragment in BIOPEP biologically active peptide database

Fig. 1. An example of the profile of potential ACE inhibitory activity performed for the chicken (*Gallus gallus*) connectin 1 (BIOPEP ID-1118)

teins contained the largest number of ACE inhibitors regarding the length of the protein chain (*16*). Figs. 2 and 3 show the newly BIOPEP-generated profiles of the ACE inhibitors in the α-lactalbumins derived from different sources and different variants of bovine β-casein, respectively.

Generally, in the case of α-lactalbumins, from seven out of eight species analyzed, the number of potential ACE inhibitors was comparable (from 38 to 45). The bovine precursor of lactalbumin (ID-1115) possessed the largest number of potential ACE inhibitors (45). The smallest number (31) of peptides lowering blood pressure was found in guinea pig (*Cavia porcellus*). Peptides consisting of two amino acid residues dominated in these proteins, but also the presence of longer fragments such as LAHKAL, WLAHK and VGINYWLAHK was observed.

Fig. 3 shows the profiles of ACE inhibitors in genetic variants of bovine β-casein (*Bos taurus*). The entire sequence of casein consists of 209 amino acid residues. All genetic variations are highlighted in red. The change of serine for arginine at position 122 of the casein chain in genetic variant B contributed to the increase of the number of potential ACE inhibitors. Variant B of casein

α-lactalbumin:

Human (*Homo sapiens*) – BIOPEP ID 1077

1. KQFTKCELSQLLKDI**DGYGGIA**LPELICT**MF**HT**SGY**D**TQ**AI**VE**NNES**TEYGLF**QIS**NKLW**CKSSQ**VPQ**SRNICDISCD**KF**LDDDLTDDIMC**AKK**ILDI**KG**

101. **I**D**YWLAHKAL**C**TEKL**EQWLC**EKL** *Total number of ACE inhibitory fragments=41*

Horse (*Equus caballus*) – BIOPEP ID 1078

1. KQFTKCQLSQVLKSM**DGYKGV**TLP**EW**ICT**IF**HN**SGY**D**TQ**TI**VK**N**NGKTEYGLF**QIN**NK**MWCRDNQ**ILP**SRNIC**GI**SC**NKF**LDDDLTDDVMC**AKK**ILDS**EG**

101. **I**D**YWLAHKPL**CS**EKL**EQWLCEEL *Total number of ACE inhibitory fragments=38*

Goat (*Capra hircus*) – BIOPEP ID 1079

1. EQLTKC**EVFQKL**KDLKD**YGGV**SLP**EW**VCT**AF**HT**SG**YD**TQ**AIVQNNDS**TEYGLF**QIN**NK**I**W**CKDDQN**PH**SRNICNISCD**KF**LDDDLTDDIVC**AKK**ILDK**VG**

101. **INYWLAHKAL**CS**EKL**DQWLC**EKL** *Total number of ACE inhibitory fragments=40*

Red-necked wallaby (*Macropus rufogriseus*) – BIOPEP ID 1081

1. IDYRKCQASQIL**KEHGM**DKV**IPLP**ELVCT**MF**HI**SGL**S**TQ**A**EV**NNHS**NKEYGIF**QISN**DGW** CA**EK**QEDVANSVC**GI**LCS**KF**LDDDITDD**IE**C**AKK**I**LQ**LP**E**

101. **GLGYWKAH**ETFCLEDLDQWRC *Total number of ACE inhibitory fragments=32*

Sheep (*Ovis aries*) – BIOPEP ID 1082

1. EQLTKC**EAFQKL**KDLKD**YGGV**SLP**EW**VCT**AF**HT**SGY**D**TQ**AIVQNNDS**TEYGLF**QIN**NKIW**CKDDQN**PH**SRNICNISCD**KF** LDDDLTDDIVC**AKK**ILDK**VG**

101. **INYWLAHKA**L CS**EKL**DQWLC**EKL** *Total number of ACE inhibitory fragments=42*

Guinea pig (*Cavia porcelluss*) – BIOPEP ID 1083

1. KQLTKCALSHE**LN**D**LAGY**RDITLP**EW**LCI**LF**HI**SGY**D**TQ**AI**VK**NSD**HKEYGLF**QINDKDFCESSTTVQSRNICDISCD**KL**LDDDLTDDIMC**VK**KILDI**KG**

101. **I**D**YWLAHKPL**CSD**KL**EQWYC**EA**Q *Total number of ACE inhibitory fragments=31*

Arabian camel (*Camelus dromedarius*) – BIOPEP ID 1085

1. KQFTKC**KL**SDELKDM**NGHGGI**T**LAEW**ICI**IF**HM**SGY**D**TE**TVVSN**NG**NR**EYGLF**QIN**NKIW**CRDNEN**LQ**SRNICDISCD**KF** LDDDLTDDKMC**AKK**ILD**KEG**

101. **I**D**YWLAHKPL**CS**EKL**EQWQC**EKW** *Total number of ACE inhibitory fragments=38*

Bovine (*Bos taurus*) [precursor] – BIOPEP ID 1115

1. MM**SF**VSLLL**V GILF**HATQAEQLTKCE**VFR**ELKDL**KGYGGV** SLPEWVCTTFHT**SGY**DTQAIVQNNDST**EYG LF**QIN**NKIW**CKDDQN**PH**SSNICNISCD**KF**L

101. DDDLTDDIMC**VK**KILDK**VGINYWLAHKAL**CS**EKL**DQWLC**EKL** *Total number of ACE inhibitory fragments=45*

ACE inhibitory fragments: **AKK** [ID-3379][1], **ILP** [ID-2642], **MF** [ID-3385], **LW** [ID-3389], **YW** [ID-3488], **LAHKAL** [ID-3514], **GY** [ID-3532], **LF** [ID-3551], **YG** [ID-3553], **YGLF** [ID-3554], **WLAHK** [ID-3970], **YGL** [ID-3973], **IA** [ID-7585], **LA** [ID-3524], **VP** [ID-7587], **GI** [ID-7596], **GL** [ID-7599], **KG** [ID-7604], **GG** [ID-7616], **SG** [ID-7618], **YGG** [ID-7647], **NKL** [ID-7654], **DG** [ID-7681], **KF** [ID-7692], **KL** [ID-7693], **NK** [ID-7698], **KA** [ID-7743], **EY** [ID-7752], **VE** [ID-7829], **TE** [ID-7830], **TQ** [ID-7834], **AH** [ID-7835], **PQ** [ID-7837], **EK** [ID-7840], **HK** [ID-7844], **PL** [ID-7513], **VK** [ID-7558], **IF** [ID-7593], **GV** [ID-7608], **GK** [ID-7611], **EG** [ID-7622], **NG** [ID-7624], **GYK** [ID-7646], **YK** [ID-7697], **KP** [ID-7810], **EW** [ID-7838], **VF** [ID-3384], **VGINYWLAHK** [ID-3971], **IW** [ID-7544], **AF** [ID-7583], **VG** [ID-7594], **QK** [ID-7680], **NY** [ID-7682], **EV** [ID-7828], **PH** [ID-7843], **PLP** [ID-2664], **GW** [ID-7579], **IP** [ID-7581], **GM** [ID-7597], **HG** [ID-7614], **LG** [ID-7619], **IE** [ID-7827], **LQ** [ID-7831], **KE** [ID-7841], **EA** [ID-7623], **AG** [ID-7600], **LN** [ID-7832], **KW** [ID-3386], **FR** [ID-7592], **SF** [ID-7685]

[1]ID number of the fragment in BIOPEP biologically active peptide database

Fig. 2. An example of the profiles of potential ACE inhibitory activity performed for the α-lactalbumins derived from different sources

β-casein:

Genetic variant A1 – BIOPEP ID 1097

1. RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQ TEDELQDKIH PFAQTQSLVY PFPGPIH[1]NSL PQNIPPLTQT PVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=114*

Genetic variant A2 – BIOPEP ID 1098

1. RELEELNVPGEIVESLSSSEESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY PFPGPIPNSL PQNIPPLTQT PVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=118*

Genetic variant A3 – BIOPEP ID 1099

1. RELEELNVPG EIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQP EVMGVSKVKE
101. AMAPKQKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQP LPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=114*

Genetic variant B – BIOPEP ID 1100

1. RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIH PFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVEPFTERQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=121*

Genetic variant C – BIOPEP ID 1101

1. RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEKQQQTEDELQDKIH PFAQTQSLVYPFPGPIHNSL PQNIPPLTQT PVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=114*

Genetic variant E – BIOPEP ID 1102

1. RELEELNVPGEIVESLSSSEESITRINKKIEKFQSKEQQQTEDELQDKIH PFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=119*

Genetic variant F – BIOPEP ID 1103

1. RELEELNVPGEIVESLSSSEESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKE
101. AMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPPLLLQSWMHQPHQP LLPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGP
201. VRGPFPIIV *Total number of ACE inhibitory fragments=112*

ACE inhibitory fragments: **VLP** [ID-2653][2], **PLP** [ID-2664], **LHLP** [ID-2668], **NLHLP** [ID-2671], **LVYP** [ID-3331], **SLVYP** [ID-3332], **TQSLVYP** [ID-3333], **QTQSLVYP** [ID-3334], **AQTQSLVYP** [ID-3335], **FAQTQSLVYP** [ID-3336], **HPFAQTQSLVYP** [ID-3337], **IHPFAQTQSLVYP** [ID-3338], **KIHPFAQTQSLVYP** [ID-3339], **KYPVQPFTESQSLTL** [ID-3350], **LPQNIPPLTQTPVVVPPFLQPEVMGVSK** [ID-3353], **RDMPIQAF** [ID-3354], **LLYQQPVLGPVRGPFPIIV** [ID-3355], **YQQPVLGPVR** [ID-3369], **AVP** [ID-3370], **AVPYP** [ID-3371], **PYP** [ID-3372], **PQR** [ID-3373], **LY** [ID-3381], **MF** [ID-3385], **LPP** [ID-3391], **LQSW** [ID-3397], **YPVQPFTE** [ID-3401], **QSLVYP** [ID-3426], **AVPYPQR** [ID-3480], **VY** [ID-3492], **SKVLPVPE** [ID-3499], **FP** [ID-3502], **TPVVVPPFLQP** [ID-3503], **VYPFPG** [ID-3504], **VYP** [ID-3505], **YQQPVL** [ID-3510], **EMPFPK** [ID-3511], **IPP** [ID-3522], **VPP** [ID-84-86], **LQP** [ID-3542], **YP** [ID-3666], **KVLPVP** [ID-7487], **VYPFPGPI** [ID-7492], **DKIHPF** [ID-7493], **LNVPGEIVE** [ID-7493], **NIPPLTQTPV** [ID-7495], **EMPFPK** [ID-7502], **LGP** [ID-7508], **GP** [ID-7512], **PL** [ID-7513], **GPV** [ID-7545], **VK** [ID-7558], **VRGPFPIIV** [ID-7566], **IP** [ID-7581], **AF** [ID-7583], **AP** [ID-7584], **VP** [ID-7587], **HL** [ID-7602], **GV** [ID-7608], **MG** [ID-7609], **GE** [ID-7615], **LG** [ID-7619], **EA** [ID-7623], **PG** [ID-7625], **VR** [ID-7628], **YPFPGPI** [ID-7665], **LLYQQPV** [ID-7665], **KY** [ID-7691], **KF** [ID-7692], **NK** [ID-7698], **KA** [ID-7743], **EI** [ID-7826], **IE** [ID-7827], **EV** [ID-7828], **VE** [ID-7829], **TE** [ID-7830], **LQ** [ID-7831], **LN** [ID-7832], **PT** [ID-7833], **TQ** [ID-7834], **PP** [ID-7836], **PQ** [ID-7837], **EK** [ID-7840], **KE** [ID-7841], **HP** [ID-7842], **PH** [ID-7843], **HK** [ID-7844], **YPFPGPIN** [ID-7486], **YPFPGPIP** [ID-7501], **LVYPFPGPIPNSLPQNIPP** [ID-7564], **LPLP** [ID-2665], **HLPLP** [ID-2666], **LHLPLP** [ID-2667], **NLHLPLP** [ID-2669], **ENLHLPLP** [ID-2672], **KVLPVPQ** [ID-3498], **KVLPVPQ** [ID-7565], **YPFPGPI** [ID-7665], **YQEPVL** [ID-7662], **QK** [ID-7680]

[1]differences between genetic variants

[2]ID number of the fragment in BIOPEP biologically active peptide

Fig. 3. Examples of the profile of potential ACE inhibitory activity performed for different genetic variants of bovine (*Bos taurus*) β-caseins

was the best source of ACE inhibitors (121). The smallest number of peptides was observed in variant F, where proline was changed for leucine at position 152 of the protein chain. Proline is the most common C-terminal residue occurring in ACE inhibitors and, with other cyclic or aromatic amino acids, is involved in the activity of the peptide (*19*). Apart from short chain fragments, caseins were in the group of proteins containing the longest peptides known as ACE inhibitors, such as *e.g.* LPQNIPPLTQTPVVVPPFLQPEVMGVSK, KYPVQPFTESQSLTL, KIHPFAQTQSLVYP and LVYPFPGPIPNSLPQNIPP.

The profiles of the biological activity of the analyzed proteins showed that some of them had not been known so far as endogenous ACE inhibitors (*e.g.* chicken myosin). This confirms the hypothesis by Karelin *et al.* (*25*) that, regardless of their basic functions, proteins can be the precursors of peptides with different types of bioactivity.

According to Dziuba and Iwaniak (*14*), the profile of potential biological activity of a protein gives an answer to the following question: are there any bioactive fragments that may occur in a protein? If the answer is positive, it suggests that protein may be a precursor of biopeptides, but we need to be aware of metabolic processes in the body. The enzymatic action of specific enzymes can contribute to the release of peptides with (not necessarily) ACE inhibitory activity. Fukudome *et al.* (*26*) showed that the opioid activity of gluten hydrolysates varied depending on the applied hydrolytic enzyme. Adequate proteolysis can facilitate the release of bioactive peptides and their bioactivity can decrease due to exceeding a certain level of hydrolysis (*15*). The effectiveness of peptide action can also be dependent on their hydrolysis resistance in the digestive tract, as well as on blood absorption (*27*). To predict the possibility and number of released peptides after the action of different proteolytic enzymes, Dziuba and Iwaniak (*14*) developed an option called 'The Enzymes' Action', which is available in the BIOPEP database. There are many bioinformatic tools predicting enzymatic hydrolysis of proteins and they are based on different algorithms. They have become popular in proteolytic process design (*28*,*29*).

Table 1 shows the values of the frequency of the occurrence of ACE inhibitors (A) as well as the potential ACE inhibitory activity of protein fragments (B). The values of A are given in ascending order. The relatively highest values of A discriminant were obtained for milk and meat proteins: bovine β-caseins (*Bos taurus*) (A in the range from 0.536 to 0.569), bovine tropoelastin (*Bos taurus*) (A=0.885), and chicken (*Gallus gallus*) and bovine (*Bos taurus*) collagens (A=0.751 and 0.874, respectively). The lowest values of the frequency of occurrence of ACE inhibitory fragments appeared in plant proteins from rice and wheat, or animal origin sequences such as lysozymes and short-chained meat proteins (*e.g.* from chicken). The exception was the relatively high value of A calculated for monellin (blueberry) (A=0.511) and wheat glutenin (A=0.558). This could be explained by the overlapping sequences of peptides (monellin) and the length of the protein chain (glutenin).

The values of potential biological activity (B), defined as the sum of the individual ACE inhibitory fragments within the above-mentioned protein sequences were: 0.0176–0.0194 (β-caseins), 0.0209 (bovine tropoelastin), 0.2340 (chicken collagen) and 0.2720 (bovine collagen).

There is no simple relationship between the frequency of occurrence of bioactive fragments in a given protein and its potential biological activity. For example, the frequency of the occurrence of ACE inhibitors in collagens is over threefold higher than in rice prolamins (Table 1). However, the potential ACE inhibitory activity of collagens is much higher, compared to rice prolamins. This results from the fact that fragments showing ACE inhibitory activity found in rice prolamins are more potent inhibitors of ACE than the corresponding fragments of collagens.

In conclusion, the higher the value of A, the higher the probability of enzymatic release of peptides from their protein precursors. In turn, lower values of B suggest that fragments present in a protein are more potent.

Table 1. The frequency of the occurrence of ACE inhibitory fragments (A) and potential ACE inhibitory activity (B) in the analyzed protein sequences

No.	Protein	A	B/μM^{-1}
1.	rice 10KD prolamin [precursor], ID-1168	0.240	0.0015
2.	13 kDa prolamin [precursor], rice, ID-1550	0.250	0.0170
3.	α-lactalbumin, guinea pig, ID-1083	0.252	0.0060
4.	rice prolamin [precursor], CLONE PPROL 17, ID-1153	0.262	0.0148
5.	α-lactalbumin, red-necked wallaby, ID-1081	0.264	0.0083
6.	tropomyosin 1 α chain, chicken, ID-1128	0.278	0.0031
7.	legumin A [precursor] (β-globulin), ID-1164	0.286	0.0044
8.	α/β-gliadin [precursor] (prolamin) (class A-I), wheat, ID-1177	0.290	0.0075
9.	α/β-gliadin [precursor] (prolamin), wheat, ID-1365	0.292	0.0065
10.	lysozyme [precursor], silk moth, ID-1093	0.292	0.0111
11.	purothionin A-I [precursor] (β-purothionin), wheat, ID-1173	0.294	0.0052
12.	lysozyme C, dog, ID-1094	0.294	0.0126
13.	lysozyme C, domestic pigeon, ID-1095	0.299	0.0099
14.	sorghum kafirin PGK1 [precursor], ID-1149	0.301	0.0043
15.	α/β-gliadin [precursor] (prolamin) (clone PW8142), wheat, ID-1184	0.304	0.0083
16.	γ-gliadin, wheat, ID-1318	0.304	0.0064
17.	C troponin skeletal muscles, chicken, ID-1133	0.304	0.0066
18.	α/β-gliadin [precursor] (prolamin) (class A-IV), wheat, ID-1183	0.306	0.0084
19.	α/β-gliadin [precursor] (prolamin) (class A-II), wheat, ID-1178	0.306	0.0049
20.	γ-gliadin, wheat, ID-1320	0.306	0.0048

Table 1. – continued

No.	Protein	A	B/μM^{-1}
21.	tropomyosin β-chain, chicken, ID-1130	0.306	0.0040
22.	α/β-gliadin [precursor] (prolamin) (class A-V), wheat ID-1181	0.307	0.0080
23.	α-2-purothionin [precursor], wheat, ID-1174	0.309	0.0046
24.	α-lactalbumin, Arabian camel, ID-1085	0.309	0.0167
25.	α-lactalbumin, horse, ID-1078	0.309	0.0088
26.	α/β-gliadin [precursor] (prolamin) (class A-III), wheat, ID-1180	0.312	0.0052
27.	α/β-gliadin fragment (prolamin) (clone PTO A10), wheat, ID-1186	0.312	0.0057
28.	γ-gliadin, wheat, ID-1317	0.312	0.0034
29.	12S seed storage globulin [precursor], oat, ID-1167	0.315	0.0133
30.	α-hordothionin [precursor] (purothionin II), barley, ID-1176	0.315	0.0035
31.	lysozyme C [precursor], chicken, ID-1092	0.315	0.0127
32.	α-lactalbumin, cow, ID-1115	0.317	0.0095
33.	sorghum kafirin PSK8 [precursor], ID-1197	0.322	0.0043
34.	α/β-gliadin [precursor] (prolamin) (MM1), wheat, ID-1179	0.322	0.0067
35.	γ-gliadin [precursor] (class B-III), wheat, ID-1148	0.322	0.0091
36.	retinol binding protein RBP, cow, ID-1198	0.322	0.0066
37.	κ-casein, goat, ID-1109	0.323	0.0050
38.	11S globulin seed storage protein G3 [precursor], common sunflower, ID-1163	0.324	0.0098
39.	α-1-purothionin [precursor] (fragment), wheat, ID-1171	0.325	0.0101
40.	γ-gliadin, wheat, ID-1319	0.325	0.0051
41.	α-lactalbumin, sheep, ID-1082	0.325	0.0120
42.	α-lactalbumin, goat, ID-1079	0.325	0.0129
43.	flavodoxin mutant D58P, ID-1108	0.326	0.0229
44.	OBP, odorant binding protein, cow, ID-1193	0.327	0.0102
45.	sorghum kafirin PSKR2 [precursor], ID-1196	0.330	0.0087
46.	legumin chain B fragment, broad bean, ID-1142	0.331	0.0011
47.	β-globulin B (legumin), ID-1141	0.332	0.0124
48.	α-lactalbumin, human, ID-1077	0.333	0.0089
49.	chicken connectin (titin), fragment, chicken, ID-1076	0.333	0.0104
50.	α/β-gliadin [precursor] (prolamin) (clone PW 1215), wheat, ID-1182	0.334	0.0059
51.	legumin J, garden pea, ID-1144	0.334	0.0091
52.	soybean basic 7S subunit globulin [precursor], ID-1162	0.340	0.0150
53.	lysozyme C, human [precursor], ID-1091	0.346	0.0119
54.	γ-hordein 1 [precursor], barley, ID-1150	0.348	0.0054
55.	11S globulin (β subunit) pumpkin, ID-1161	0.350	0.0129
56.	cocoa storage protein, ID-1114	0.351	0.0088
57.	γ-1-purothionin, wheat, ID-1172	0.362	0.0029
58.	κ-casein gen. var. A, cow, ID-1117	0.363	0.1563
59.	α-S_2-casein gen. var. A, cow, ID-1090	0.365	0.0099
60.	troponin T, cardiac muscle isoforms, cow, ID-1273	0.368	0.1100
61.	rice prolamin [precursor] CLONE PPROL 14, ID-1154	0.372	0.0230
62.	troponin C, pig, ID-1134	0.375	0.0074
63.	human lactoferrin, ID-1121	0.377	0.0158
64.	γ-hordothionin, barley, ID-1175	0.383	0.0111
65.	legumin 11S globulin, ginko, ID-1143	0.384	0.0105
66.	bilin binding protein BBP, white butterfly, ID-1199	0.386	0.0203
67.	rice prolamin [precursor], CLONE PPROL 4A, ID-1155	0.387	0.0223
68.	myosin light chain, chicken (I), ID-1122	0.395	0.0047
69.	Che Y, chemotactic protein (*Escherichia coli*), ID-1106	0.403	0.0137
70.	rice prolamin [precursor], CLONE PPROL 7, ID-1152	0.409	0.0240
71.	legumin-like protein, mouse-ear cress, ID-1140	0.412	0.0130
72.	blueberry monellin, B chain, ID-1169	0.420	0.0217
73.	phycocyanin, ID-1126	0.420	0.0101
74.	β-lactoglobulin, cow, gen. var. A, ID-1116	0.421	0.0269
75.	plastocyanin, rice, ID-1564	0.422	0.0059
76.	κ-casein, human, ID-1120	0.456	0.0114
77.	α-S_1-casein gen. var. A, cow, ID-1086	0.462	0.0111
78.	α-S_1-casein gen. var. B, cow, ID-1087	0.467	0.0144
79.	α-S_1-casein gen. var. D, cow, ID-1089	0.467	0.0139
80.	β-lactoglobulin, sheep, ID-1105	0.469	0.0295
81.	α-S_1-casein gen. var. C, cow, ID-1088	0.472	0.0143
82.	β-lactoglobulin, goat, ID-1104	0.475	0.0295
83.	epidermal retin acid binding protein EBP, mouse-ear cress, ID-1200	0.491	0.0193
84.	blueberry monellin, chain A, ID-1170	0.511	0.0214
85.	β-casein gen. var. F, cow, ID-1103	0.536	0.0176
86.	β-casein gen. var. A^1, cow, ID-1097	0.545	0.0180
87.	β-casein gen. var. A^3, cow, ID-1099	0.545	0.0180
88.	β-casein gen. var. C, cow, ID-1101	0.550	0.0180
89.	wheat glutenin, high molecular mass subunit PW212 [precursor], ID-1110	0.558	0.0190
90.	β-casein gen. var. B, cow, ID-1100	0.558	0.0194
91.	β-casein gen. var. A^2, cow, ID-1098	0.565	0.0193
92.	β-casein gen. var. E, cow, ID-1102	0.569	0.0193
93.	collagen α-1(I) chain [precursor], chicken, ID-1113	0.751	0.2340
94.	collagen α-1(III) chain, cow, ID-1111	0.854	0.2740
95.	collagen α-1(I) chain (fragment), cow, ID-1112	0.874	0.2720
96.	elastin [precursor] (tropoelastin), cow, ID-1107	0.885	0.0209

Table 2 presents the idea of comparison of the major groups of proteins as precursors of ACE inhibitors. A statistical *t*-test for independent variables was performed using Statistica v. 8 software. For example, the values of A for one group of proteins were compared with A for another group of proteins. The same rule referred to potential ACE inhibitory activity (B values of one group *vs.* B for another group). All statistically significant differences were obtained at $p<0.05$. Significant differences were not observed between the following groups: wheat gliadins *vs.* chicken troponins, wheat gliadins *vs.* chicken meat proteins, rice proteins *vs.* chicken meat proteins. Mean potential ACE inhibitory activity (B) suggests that β-lactoglobulins are a source of less potent ACE inhibitory peptides than soy globulins. The same conclusion refers to the wheat gliadins *vs*. rice prolamins. The mean frequencies of the occurrence of ACE inhibitors in the above-mentioned groups of proteins are approximately at the same level. The *t*-test confirmed the results in Table 1: *e.g.* although β-caseins are a better potential source of ACE inhibitors than α-S_1-caseins (significance at $p<0.05$), the latter possess more potent peptides with this activity. β-Lactoglobulins are a richer source of ACE inhibitory peptides than α-lactalbumins from different sources, lysozymes or α/β-wheat gliadins (and all analyzed gliadins).

Comparing the above-mentioned three criteria of protein evaluation, it can be said that although the potential ACE inhibitory activity of protein (B) is a more accurate measure of protein value, the other two criteria such as the profile of potential ACE inhibitory activity of protein as well as the frequency of the occurrence of fragments with ACE inhibitory activity (A) can be used for that purpose more widely. This is due to the fact that the calculation of B requires information about the IC_{50} (the concentration required to inhibit 50 % of the ACE activity). The values of the above-mentioned measure may depend on the assay. Murray *et al.* (*21*) found that the IC_{50} values obtained for a known ACE inhibitor, captopril, ranged from 5 to 23 nM using HHL and from 1.61 to 8.91 nM when using FAPGG to determine ACE activity. Detailed information on the number of enzyme units applied in all ACE inhibition analyses seems to be essential for comparable purposes. The comparison of IC_{50} values obtained for different substances can be more meaningful if the IC_{50} value for a standard like captopril is reported (*21*).

Despite these obstacles, the *in silico* methods applied in the determination of protein value as the potential precursors of peptides with various activities can be a preliminary 'qualifier' of protein in functional food manufacturing, especially nutraceuticals, *i.e.* food with special therapeutic properties (*5*). Moreover, detection of functional motifs (*i.e.* ACE inhibitory fragments) in a protein sequence can reveal the protein's function and, consequently, it can be assigned to the evolutionarily related protein family (*28*). Dziuba *et al.* (*30*) analyzed bovine β-lactoglobulin and other protein sequences from the lipocalin family and found some similar domains containing fragments with different bioactivities, including ACE inhibitory ones. This confirms the contemporary approach that proteins with similar sequences and spatial structures can play different roles in living systems (*31*). This rule may also be applied to proteins as the source of ACE inhibitory peptides.

Conclusions

The criteria for protein evaluation presented in this paper are suitable measures to identify which plant or animal proteins could serve as a potentially good source of peptides with ACE inhibitory activity. The profile of

Table 2. The results of *t*-test for various protein groups

Proteins		Mean A		Mean B		*t*-value	Significant at p
Group 1 *vs.* Group 2		Group 1	Group 2	Group 1	Group 2		
β-caseins	α-S_1-caseins	0.552	0.465	–	–	12.148	<0.05
		–	–	0.0185	0.0131	6.983	<0.05
β-lactoglobulins	α-lactalbumins	0.455	0.304	–	–	7.496	<0.05
		–	–	0.0286	0.0103	8.907	<0.05
β-lactoglobulins	lysozymes	0.455	0.310	–	–	7.788	<0.05
		–	–	0.0286	0.0100	9.658	<0.05
β-lactoglobulins	α/β-wheat gliadins	0.455	0.313	–	–	12.619	<0.05
		–	–	0.0286	0.0060	22.512	<0.05
β-lactoglobulins	wheat gliadins	0.455	0.310	–	–	15.038	<0.05
		–	–	0.0286	0.0060	21.978	<0.05
soybean globulins	β-lactoglobulins	0.340	0.455	–	–	–3.360	–
		–	–	0.0150	0.0064	5.164	<0.05
wheat gliadins	chicken troponins	0.310	0.304	–	–	0.514	–
		–	–	0.0639	0.0066	–0.124	–
wheat gliadins	chicken meat proteins	0.310	0.383	–	–	–1.737	–
		–	–	0.0639	0.0393	–1.530	–
rice proteins	wheat gliadins	0.334	0.310	–	–	1.256	–
		–	–	0.155	0.0639	3.977	<0.05
rice proteins	chicken meat proteins	0.334	0.383	–	–	–0.659	–
		–	–	0.155	0.0393	–0.730	–

the potential biological activity of a protein, as well as the frequency of the occurrence of fragments with biological activity (A) can be applied to evaluate proteins as a source of peptides with other activities. These results confirmed that genetic variants of bovine caseins are the best sources of ACE inhibitors. Among the other analyzed sequences, it was found that soy globulins can be a better source of these peptides than bovine β-lactoglobulins. All wheat gliadins gathered in BIOPEP can be better precursors of ACE inhibitory fragments than chicken meat proteins. However, it needs to be considered that: (*i*) proteolytic processes determine the potential release of such peptides from the protein; (*ii*) the reliability and accuracy of *in silico* methods in protein evaluation is often subjective due to discretion in the interpretation of the results (depending on the different algorithms applied in biochemistry, molecular biology, comparative modeling); and (*iii*) to date, unequivocal rules in protein classification have not been established.

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