ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Technol. Aliment. 8(1) 2009, 71-90*

MILK PROTEINS AS PRECURSORS OF BIOACTIVE PEPTIDES

Marta Dziuba, Bartłomiej Dziuba, Anna Iwaniak
University of Warmia and Mazury in Olsztyn

Abstract. Milk proteins, a source of bioactive peptides, are the subject of numerous research studies aiming to, among others, evaluate their properties as precursors of biologically active peptides. Physiologically active peptides released from their precursors may interact with selected receptors and affect the overall condition and health of humans. By relying on the BIOPEP database of proteins and bioactive peptides, developed by the Department of Food Biochemistry at the University of Warmia and Mazury in Olsztyn (www.uwm.edu.pl/biochemia), the profiles of potential activity of milk proteins were determined and the function of those proteins as bioactive peptide precursors was evaluated based on a quantitative criterion, i.e. the occurrence frequency of bioactive fragments (A). The study revealed that milk proteins are mainly a source of peptides with the following types of activity: antihypertensive (A_{max} = 0.225), immunomodulating (0.024), smooth muscle contracting (0.011), antioxidative (0.029), dipeptidyl peptidase IV inhibitors (0.148), opioid (0.073), opioid antagonistic (0.053), bonding and transporting metals and metal ions (0.024), antibacterial and antiviral (0.024), and antithrombotic (0.029). The enzymes capable of releasing bioactive peptides from precursor proteins were determined for every type of activity. The results of the experiment indicate that milk proteins such as lactoferrin, α-lactalbumin, β-casein and κ-casein hydrolysed by trypsin can be a relatively abundant source of biologically active peptides.

Key words: bioactive peptides, milk proteins, *in silico* proteolysis

INTRODUCTION

Milk proteins and other food proteins are analysed mainly as a source of amino acids indispensable for proper bodily functions. Other evaluation criteria are also taken into account, including the consumed protein's effect on body weight, the type and content of antinutritional compounds occurring together with proteins and their allergenic properties [Bush and Hefle 1996, Friedman 1996, Fukudome and Yoshikawa 1992]. According to the present level of knowledge, in addition to its primary function, every protein may be a precursor of biologically active (bioactive) peptides. This hypothesis postu-

Corresponding author – Adres do korespondencji: Dr inż. Bartłomiej Dziuba, Department of Industrial and Food Microbiology of University of Warmia and Mazury in Olsztyn, Cieszyński Square 1, 10-957 Olsztyn, Poland, e-mail: niklema@uwm.edu.pl

lates that every protein may be a reserve source of peptides controlling the life processes of organisms [Karelin et al. 1998]. A new, additional criterion for evaluating proteins as a potential source of biologically active peptides has been proposed [Dziuba et al. 1999 a]. Biologically active peptides in the protein sequence are defined as fragments that remain inactive in precursor protein sequences, but when released, for example by proteolytic enzymes, they may interact with selected receptors and regulate the body's physiological functions. The effect exerted by such peptides may be positive or negative [Schlimme and Meisel 1995, Meisel and Bockelmann 1999].

Milk proteins are the best researched precursors of biologically active peptides [Meisel 1998, Pihlanto-Leppälä 2001, Dziuba et al. 1999 b, 2002, Gobbetti et al. 2002, Kilara and Panyam 2003, Schanbacher et al. 1997]. Casein and whey proteins are rich in motifs exhibiting antihypertensive, opioid, antibacterial and immunomodulating activity. Proteases naturally occurring in food products, such as milk plasmin, hydrolyse proteins and release bioactive fragments during processing or storage. Many types of bacteria applied in the production of fermented food and occurring naturally in the gastrointestinal tract are capable of producing biologically active peptides. Cheese contains phosphopeptides which are further proteolysed in the process of cheese ripening, leading to the formation of various ACE inhibitors [Saito et al. 2000]. In a study of industrial cultures of milk fermenting bacteria, Pihlanto-Leppälä et al. [1998] concluded that the investigated bacteria do not form ACE inhibitor peptides from casein or whey proteins and that they are released during continued proteolysis. The results of a study of lactic acid bacteria (*Lactobacillus* subsp.) which are present in fermented dairy products, but which are not found in starter cultures, as well as in the human digestive tract indicate that their proteolytic ability is comparable to that of *Lactococcus lactis*. Proteinases found in the cell walls of *Lactococcus lactis* (PI and PIII) catalyse the first stage of casein hydrolysis. Proteinase PI is β-casein-specific, and proteinase PIII is α_s--casein and β-casein-specific. The above findings have been validated by many research teams [Juillard et al. 1995]. The short sections of both hydrolysable forms of casein create fragments containing up to 10 amino acid residues. Casein-derived peptides make up a populous group, and those fragments correspond to bioactive peptide sequences. Fragments of β-casein 60-68 and 190-193 correspond to sections of β-casomorphin-11 and immunopeptide, respectively [Korhonen and Pihlanto-Leppälä 2001]. Several casokinins have also been obtained as a result of the effect that serine proteinase from *Lactobacillus helveticus* CP790 has on β-casein, while milk fermentation with the involvement of starter cultures containing *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae* led to the formation of β-casokinin. An antihypertensive fragment of β-casein (residues: 169-175, KVLPVPE) was isolated from a casein hydrolysate with the application of extracellular proteinase from *Lactobacillus helveticus*. This peptide exhibited weak ACE inhibitor activity (IC_{50} > 1000 μmol/l). A corresponding hexapeptide with KVLPVP sequence, obtained after splitting off the C-terminal glutamine residue (E), displayed much stronger antihypertensive activity (IC_{50} = 5 μmol/l) [Meisel and Bockelmann 1999]. ACE inhibitor peptides were also obtained from milk fermented by *Lactobacillus delbruecki* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4 bacteria [Gobbetti et al. 2000].

In addition to analytical methods, many research laboratories resort to computer-aided techniques for evaluating food components, including proteins. The process of modeling the physical and chemical properties of proteins [Lackner 1999], predicting

their secondary structure [Bairoch and Apweiler 2000] or searching for a homology between proteins to identify their functions [Kriventseva et al. 2001, Bray et al. 2000] requires analyses supported by databases of protein sequences or sequence motifs [Bennett et al. 2004, Colinge and Masselot 2004]. A complementary part of such research is the strategy of examining proteins and bioactive peptides.

The objective of this study was to evaluate milk proteins as bioactive peptide precursors based on a profile of potential biological activity, the occurrence frequency of bioactive fragments in the protein sequence and the possibility of bioactive peptide release by proteolytic enzymes.

MATERIALS AND METHODS

The evaluation of milk proteins as bioactive peptide precursors and their *in silico* proteolytic release was carried out based on the BIOPEP database of proteins and bioactive peptides, developed by the Department of Food Biochemistry (www.uwm.edu.pl/biochemia). A total of 23 types of activity were analysed: antiamnestic, antithrombotic, antihypertensive, immunomodulating, chemotactic, contracting, toxic, embryotoxic, antioxidative, dipeptidyl peptidase IV inhibiting, opioid and opioid antagonistic, stimulating red blood cell formation, hemolytic, binding and transporting metals and metal ions, bacterial permease ligand, anorectic, activating ubiquitin-mediated proteolysis, regulating ion flow, neuropeptide inhibiting, regulating gastric mucosa activity, antibacterial, antiviral, regulating phosphoinositol function. Peptides with the investigated types of activity were selected in view of the frequency of their occurrence and other health and technological properties. The experiment involved 16 protein amino acid sequences from the BIOPEP database.

Functions of the BIOPEP application

The following analytical functions are available in the "Analysis" window of the BIOPEP application: developing a list of proteins or bioactive peptides with a given type of activity based on the "List of proteins" or "List of peptides with given activity" option; determining the type, number and location of active protein fragments – identifying the peptide profile ("Profiles of protein's biological activity"); computing parameters A, B and Y to determine the value of a given protein as a source of bioactive peptides ("A, B, Y Calculation"); performing *in silico* proteolysis with the use of the "Enzyme action" option. The value of proteins as bioactive peptide precursors was evaluated based on the occurrence frequency of bioactive fragments in the protein chain (A) defined as:

$$A = \frac{a}{N}$$

where:

a – number of fragments with given activity in the protein chain,
N – number of amino acid residues in the polypeptide chain of a protein molecule.

In silico proteolysis of milk proteins

The *in silico* proteolysis of milk proteins was carried out with the use of a single enzyme (24 enzymes). The BIOPEP database contains information on the following 24 proteolytic enzymes: chymotrypsin A, trypsin, pepsin, proteinase K, pancreatic elastase, propyl oligopeptidase, V-8 protease (glutamyl endopeptidase), thermolysin, plasmin, cathepsin G, clostripain, chymase, papain, ficain, leukocyte elastase, chymotrypsin C, metridin, thrombin, bromelain, pancreatic elastase II, glutamyl endopeptidase II, oligopeptidase B, calpain and glycyl endopeptidase.

Verification of results by mass spectrometry

The molecular mass and amino acid sequences of peptides released by proteolytic enzymes were studied by matrix-assisted laser desorption/ionization mass spectrometry with the use of an Ettan MALDI-ToF Pro (Amersham Biosciences) mass spectrometer. For the purpose of determining the molecular mass of the released peptides and their identification, trypsin hydrolysates of selected proteins were analysed in reflectron mode by PMF (peptide mass fingerprinting) analysis. The positive ions analysis function and 20 kV accelerating voltage were used. Samples were prepared by the dried-droplet method. Proteins were hydrolysed in an ammonium bicarbonate solution (pH of 8.5) with the use of trypsin for a proteomic analysis (Sigma) at a 1:50 enzyme to substrate ratio (w/w). Hydrolysis was performed for 24 h at a temperature of 37°C. Samples were subjected to an MS analysis.

RESULTS AND DISCUSSION

The development of the BIOPEP database and its built-in software options support a comprehensive analysis of proteins and bioactive peptides to determine whether they can be derived from protein precursors. The experiment relied on *in silico* studies to evaluate proteins as precursors of bioactive peptides as well as on a computer-aided simulation of the proteolysis process. The obtained results have to be verified by analytical methods such as two-dimensional electrophoresis, high performance liquid chromatography (HPLC) and mass spectrometry.

Tables 1-6 present the biological activity profiles of the main milk protein sequences, including the values of parameter *A* for all types of activity noted in the analysed proteins. The predominant milk protein fragments exhibit antihypertensive and dipeptidyl peptidase IV inhibiting activity. The obtained values of parameter *A* for those types of activity were relatively the highest, reaching: as regards antihypertensive activity – from 0.047 for lactoferrin and serum albumin to 0.225 for β-casein, and as regards dipeptidyl peptidase IV inhibiting activity – from 0.024 for α-lactalbumin to 0.148 for β-casein. Fragments with other types of activity are also found in milk proteins (Tables 1-6). None of the published sources account for the fact that casein contains fragments corresponding to peptides with dipeptidyl peptidase IV inhibiting activity, i.e. a proteolytic enzyme involved in digestion processes [Pereira and Ciclitira 2004]. Many peptides with the above types of activity were obtained by enzymatic hydrolysis of casein. Coste et al. [1992] relied on this method to produce fragments of β-casein (residues 193-209) with immunomodulating activity.

Table 1. Profile of potential biological activity of cow's (*Bos taurus*) α_{s1}-casein (genetic variant A) with the values of discriminants *A* – BIOPEP

Protein sequence (186 amino acid residues, ID 1086)	
RPKHPIKHQGLPQPFPQVFGKEKVNELSKDIGSESTEDQAMEDIKEMEAESISSSEEIVPNSVEQKHIQ KEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKQGIHAQQKEPMIGVNQELAYFYPE LFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.134)	
RL	[87-88], [106-107]
FGK	[19-21]
RY	[77-78]
VF	[18-19]
LW	[185-186]
AYFYP	[130-134]
YKVPQL	[91-96]
AYFYPE	[130-135]
FP	[15-16]
DAYPSGAW	[144-151]
LAYFYP	[129-134]
TTMPLW	[181-186]
PLW	[184-186]
GY	[80-81]
YL	[78-79], [81-82]
LF	[136-137]
FY	[132-133], [140-141]
LAY	[129-131]
AY	[130-131], [145-146]
YP	[133-134], [146-147]
Immunomodulating activity (A = 0.011)	
EAE	[48-50]
LGY	[79-81]
Opioid activity (A = 0.027)	
PLG	[155-157]
TTMPLW	[181-186]
YLGYLE	[78-83]
YL	[78-79], [81-82]
Opioid antagonist activity (A = 0.011)	
RYLGYLE	[77-83]
RYLGYL	[77-82]

Table 1 – cont.

1	2
Regulating the action mechanism of phosphoinositol activity (A = 0.005)	
LGY	[79-81]
Antioxidative activity (A = 0.005)	
LH	[107-108]
Ligands of bacterial permease activity (A = 0.005)	
KK	[89-90]
Antiamnestic activity (A = 0.0053)	
VPL	[154-156]
Dipeptidyl peptidase IV inhibitors activity (A = 0.070)	
MP	[183-184]
LA	[129-130]
AP	[163-164]
FP	[15-16]
LP	[11-12]
VP	[59-60], [73-74], [93-94], [99-100], [154-155]
LL	[85-86]
HA	[115-116]
Activating ubiquitin-mediated proteolysis (A = 0.005)	
LA	[129-130]

Table 2. Profile of potential biological activity of cow's (*Bos taurus*) β-casein (genetic variant A_1) with the values of discriminants *A* – BIOPEP

Protein sequence (209 amino acid residues, ID 1097)	
RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIHNSL PQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVQPFTESQSLTLTDVENLHLPP LLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPEKAVPYPQRDMPIQAFLLYQQPVLGPVRG PFPIIV	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.225)	
VLP	[170-172]
PLP	[150-152]
LHLP	[133-136]
NLHLP	[132-136]
LVYP	[58-61]
SLVYP	[57-61]
TQSLVYP	[55-61]
QTQSLVYP	[54-61]

Table 2 – cont.

1	2
AQTQSLVYP	[53-61]
FAQTQSLVYP	[52-61]
HPFAQTQSLVYP	[50-61]
IHPFAQTQSLVYP	[49-61]
KIHPFAQTQSLVYP	[48-61]
KYPVQPFTESQSLTL	[113-127]
LPQNIPPLTQTPVVVPPFLQPEVMGVSK	[70-97]
RDMPIQAF	[183-190]
LLYQQPVLGPVRGPFPIIV	[191-209]
YQQPVLGPVR	[193-202]
AVP	[177-179]
AVPYP	[177-181]
PYP	[179-181]
PQR	[181-183]
LY	[192-193]
MF	[156-157]
LPP	[135-137], [151-153]
LQSW	[140-143]
YPVQPFTE	[114-121]
QSLVYP	[56-61]
AVPYPQR	[177-183]
VY	[59-60]
SKVLPVPE	[168-175]
FP	[62-63], [111-112], [157-158], [205-206]
TPVVVPPFLQP	[80-90]
VYPFPG	[59-64]
VYP	[59-61]
YQQPVL	[193-198]
EMPFPK	[108-113]
IPP	[74-76]
VPP	[84-86]
LQP	[88-90]
YP	[60-61], [114-115], [180-181]
Antiamnestic activity (A = 0.043)	
IHPFAQTQ	[49-56]
VYPFPGPIH	[59-67]
VYPFPGPI	[59-66]
PGP	[63-65]
PG	[9-10], [63-64]
GP	[64-65], [199-200], [203-204]

Table 2 – cont.

1	2
Antithrombotic activity (A = 0.029)	
GP	[64-65], [199-200], [203-204]
PGP	[63-65]
PG	[9-10], [63-64]
Immunomodulating activity (A = 0.019)	
RELEELNVPGEIVESLSSSEESITR	[1-25]
YQQPVLGPVRGPFPIIV	[193-209]
YQQPVLGPVR	[193-202]
LLY	[191-193]
Ligands of bacterial permease activity (A = 0.005)	
KK	[28-29]
Dipeptidyl peptidase IV inhibitors activity (A = 0.148)	
PP	[75-76], [85-86], [136-137], [152-153], [158-159]
MP	[109-110], [185-186]
MA	[102-103]
KA	[176-177]
FA	[52-53]
AP	[103-104]
FP	[62-63], [111-112], [157-158], [205-206]
LP	[70-71], [135-136], [151-152], [171-172]
VP	[8-9], [84-85], [173-174], [178-179]
LL	[138-139], [139-140], [191-192]
VV	[82-83], [83-84]
GP	[64-65], [199-200], [203-204]
Opioid activity (A = 0.009)	
YPFP	[60-63]
YPF	[60-62]
Opioid antagonist activity (A = 0.009)	
YPFPGPI	[60-66]
YPFPG	[60-64]
Regulating the functions of the gastric mucosa (A = 0.029)	
GP	[64-65], [199-200], [203-204]
PG	[9-10], [63-64]
PGP	[63-65]
Antioxidative activity (A = 0.009)	
LH	[133-134]
HL	[134-135]

Table 3. Profile of potential biological activity of cow's (*Bos taurus*) κ-casein (genetic variant A) with the values of discriminants *A* – BIOPEP

Protein sequence (169 amino acid residues, ID 1117)	
QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAV RSPAQILQWQVLSDTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPT TEAVESTVATLEDSPEVIESPPEINTVQVTSTAV	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.065)	
IR	[9-10]
PYP	[57-59]
RY	[34-35]
RF	[16-17]
YIPIQYVLSR	[25-34]
IPP	[108-110]
YG	[38-39]
AIP	[107-109]
YP	[35-36], [58-59]
YGL	[38-40]
Antithrombotic activity (A = 0.024)	
NQDK	[113-116]
MAIPPKKNQDK	[106-116]
MAIPPK	[106-111]
MAIPPKK	[106-112]
MAIPPKKNQDK	[106-116]
Immunomodulating activity (A = 0.012)	
YIPIQYVLSR	[25-34]
YG	[38-39]
Opioid antagonist activity (A = 0.029)	
YG	[38-39]
YPSYGLN	[35-41]
YPYY	[58-61]
YIPIQYVLSR	[25-34]
SRYPSY	[33-38]
Antioxidative activity (A = 0.029)	
HPHL	[100-103]
PYY	[59-61]
HPH	[98-100], [100-102]
HL	[102-103]

Table 3 – cont.

1	2
Smooth muscle contracting activity (A = 0.012)	
YIPIQYVLSR	[25-34]
YVLSR	[30-34]
Ligands of bacterial permease activity (A = 0.006)	
KK	[111-112]
Dipeptidyl peptidase IV inhibitors activity (A = 0.083)	
PP	[109-110], [156-157]
VA	[48-49], [143-144]
MA	[95-96], [106-107]
PA	[64-65], [70-71], [84-85]
LP	[56-57]
VP	[83-84]

Table 4. Profile of potential biological activity of cow's (*Bos taurus*) *α*-lactalbumin (genetic variant B) with the values of discriminants *A* – BIOPEP

Protein sequence (122 amino acid residues, ID 1115)	
EQLTKCEVFRELKDLKGYGGVSLPEWVCTFHTSGYDTEAIVENNQSTDYGLFQINNKIWCKNDQD PHSSNICNISCDKFLNNDLTNNIMCVKKILDKVGINYWLAHKALCSEKLDQWLCEKL	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.098)	
VF	[8-9]
YW	[102-103]
LAHKAL	[104-109]
GY	[17-18], [34-35]
LF	[51-52]
YG	[18-19], [49-50]
YGLF	[49-52]
WLAHK	[103-107]
VGINYWLAHK	[98-107]
YGL	[49-51]
Immunomodulating activity (A = 0.024)	
YGG	[18-20]
YG	[18-19], [49-50]
Opioid activity (A = 0.024)	
YG	[18-19], [49-50]
GLF	[50-52]

Table 4 – cont.

1	2
Opioid antagonist activity (A = 0.008)	
YGLF	[49-52]
Antibacterial activity (A = 0.024)	
EQLTK	[1-5]
ALCSEK	[108-113]
ISCDKF	[74-79]
Ligands of bacterial permease activity (A = 0.008)	
KK	[92-93]
Regulating the action mechanism of phosphoinositol activity (A = 0.008)	
GLF	[50-52]
Binding and transporting metals and metal ions activity (A = 0.008)	
DY	[48-49]
Dipeptidyl peptidase IV inhibitors activity (A = 0.024)	
KA	[107-108]
LA	[104-105]
LP	[23-24]
Activating ubiquitin-mediated proteolysis activity (A = 0.008)	
LA	[104-105]

Table 5. Profile of potential biological activity of cow's (*Bos taurus*) β-lactoglobulin (genetic variant A) with the values of discriminants *A* – BIOPEP

Protein sequence (162 amino acid residues, ID 1116)	
LIVYQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENDEC AQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEA LEKFDKALKALPMHIRLSFNPTQLEEQCHI	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.136)	
YLLF	[102-105]
RL	[148-149]
IR	[147-148]
HIRL	[146-149]
HIR	[146-148]
ALPMHIR	[142-148]
VF	[81-82]
KW	[60-61]

Table 5 – cont.

1	2
LVL	[93-95]
VY	[3-4], [41-42]
IPA	[78-80]
GLDIQK	[9-14]
VAGTWY	[15-20]
LVR	[122-124]
YL	[102-103]
LF	[104-105]
LAMA	[22-25]
LDAQSAPLR	[32-40]
CMENSA	[106-111]
VLDTDYK	[94-100]
VAGTW	[15-19]
Opioid activity (A = 0.006)	
YL	[102-103]
Opioid antagonist activity (A = 0.006)	
YLLF	[102-105]
Binding and transporting metals and metal ions activity (A = 0.006)	
DY	[98-99]
Neuropeptide inhibitors (A = 0.006)	
KPT	[47-49]
Ligands of bacterial permease activity (A = 0.012)	
KK	[69-70], [100-101]
Antibacterial activity (A = 0.006)	
VAGTWY	[15-20]
Dipeptidyl peptidase IV inhibitors activity (A = 0.068)	
VA	[15-16]
MA	[24-25]
KA	[138-139], [141-142]
LA	[22-23]
AP	[37-38]
PA	[79-80]
LP	[143-144]
LL	[31-32], [57-58], [103-104]
Activating ubiquitin-mediated proteolysis activity (A = 0.006)	
LA	[22-23]

Table 6. Profile of potential biological activity of cow's (*Bos taurus*) *lactoferrin* with the values of discriminants *A* – BIOPEP

Protein sequence (689 amino acid residues, ID 1212)	
APRKNVRWCTISQPEWFKCRRWQWRMKKLGAPSITCVRRAFALECIRAIAEKKADAVTLDGGMV FEAGRDPYKLRPVAAEIYGTKESPQTHYYAVAVVKKGSNFQLDQLQGRKSCHTGLGRSAGWIIPM GILRPYLSWTESLEPLQGAVAKFFSASCVPCIDRQAYPNLCQLCKGEGENQCACSSREPYFGYSGAF KCLQDGAGDVAFVKETTVFENLPEKADRDQYELLCLNNSRAPVDAFKECHLAQVPSHAVVARSV DGKEDLIWKLLSKAQEKFGKNKSRSFQLFGSPPGQRDLLFKDSALGFLRIPSKVDSALYLGSRYLTT LKNLRETAEEVKARYTRVVWCAVGPEEQKKCQQWSQQSGQNVTCATASTTDDCIVLVLKGEADA LNLDGGYIYTAGKCGLVPVLAENRKSSKHSSLDCVLRPTEGYLAVAVVKKANEGLTWNSLKDKKS CHTAVDRTAGWNIPMGLIVNQTGSCAFDEFFSQSCAPGADPKSRLCALCAGDDQGLDKCVPNSKE KYYGYTGAFRCLAEDVGDVAFVKNDTVWENTNGESTADWAKNLNREDFRLLCLDGTRKPVTEA QSCHLAVAPNHAVVSRSDRAAHVKQVLLHQQALFGKNGKNCPDKFCLFKSETKNLLFNDNTECL AKLGGRPTYEEYLGTEYVTAIANLKKCSTSPLLEACAFLTR	
Sequence	Location in protein chain
1	2
Antihypertensive activity (A = 0.056)	
RL	[500-501], [570-571]
IR	[46-47]
FGK	[278-280], [618-620]
GRP	[653-655]
RY	[323-324], [341-342]
LY	[318-319]
IY	[81-82], [399-400]
VF	[64-65], [214-215]
LVL	[383-385]
VW	[346-347], [548-549]
HY	[91-92]
GGY	[396-398]
VAA	[77-79]
VAP	[591-593]
GY	[191-192], [397-398], [432-433], [525-526]
PR	[2-3]
LRP	[74-76], [132-134], [427-429]
IRA	[46-48]
YL	[135-136], [319-320], [324-325], [433-434], [660-661]
HY	[91-92]
YG	[82-83], [524-525]
AY	[165-166]
YP	[166-167]
Immunomodulating activity (A = 0.085)	
GFL	[306-308]
TRKP	[577-580]

Table 6 – cont.

1	2
RKP	[578-580]
RKSSK	[415-419]
YG	[82-83], [524-525]
Opioid activity (A = 0.010)	
YG	[82-83], [524-525]
YL	[135-136], [319-320], [324-325], [433-434], [660-661]
Antithrombotic activity (A = 0.004)	
GP	[351-352]
PG	[293-294], [493-494]
Anticarcinogenic activity (A = 0.003)	
FKCRRWQWRMKKLGAPSITCVRRAF	[17-41]
RRWQWR	[20-25]
Antioxidative activity (A = 0.004)	
LH	[612-613]
HL	[246-247], [588-589]
Ligands of bacterial permease activity (A = 0.010)	
KK	[27-28], [52-53], [99-100], [356-357], [440-441], [454-455], [673-674]
Antibacterial activity (A = 0.014)	
FKCRRWQWRMKKLGAPSITCVRRAF	[17-41]
APRKNVRW	[1-8]
FKCRRWQWRMKKLGAPSITCVRRAFA	[17-42]
FKCRRWQWRMKKLGAPSITCVRRAFAL	[17-43]
APRKNVRWCTISQPEW	[1-16]
CIRA	[45-48]
FKCRRWQWRMKKLGAPSITCVRRAFALECIR	[17-47]
APRKNVRWCTI	[1-11]
CRRWQWRMKKLGAPSITCV	[19-37]
FKCRRWQWRMKKLG	[17-30]
Binding and transporting metals and metal ions activity (A = 0.006)	
WQWRMKKLGA	[22-31]
PSITCVRRAF	[32-41]
APRKNVRWCT	[1-10]
FKCRRWQWRMKKLGA	[17-31]
Antiviral activity (A = 0.003)	
ADRDQYELL	[222-230]
EDLIWK	[264-269]

Table 6 – cont.

1	2
Antiamnestic activity (A = 0.0043)	
PG	[293-294], [493-494]
GP	[351-352]
Dipeptidyl peptidase IV inhibitors activity (A = 0.069)	
GQ	[294-295], [366-367]
GP	[351-352]
PP	[292-293]
VA	[77-78], [95-96], [149-150], [206-207], [256-257], [436-437], [540-541], [591-592]
KA	[53-54], [221-222], [273-274], [339-340], [441-442]
LA	[247-248], [411-412], [434-435], [533-534], [589-590], [648-649]
FA	[41-42]
AP	[1-2], [31-32], [237-238], [492-493], [592-593]
LP	[218-219]
VP	[158-159], [250-251], [408-409], [516-517]
LL	[229-230], [270-271], [298-299], [571-572], [611-612], [639-640], [680-681]
VV	[97-98], [255-256], [345-346], [438-439], [597-598]
HA	[253-254], [595-596]
Activating ubiquitin-mediated proteolysis activity (A = 0.016)	
RA	[39-40], [47-48], [236-237], [603-604]
LA	[247-248], [411-412], [434-435], [533-534], [589-590], [648-649]
WA	[560-561]
Regulating the functions of the gastric mucosa (A = 0.004)	
GP	[351-352]
PG	[293-294], [493-494]
Regulating the action mechanism of phosphoinositol activity (A = 0.001)	
GFL	[306-308]

Active fragments of milk proteins are usually made up of two or three amino acid residues. The only exception is lactoferrin which, in addition to dipeptides and tripeptides, contains fragments with up to 25 amino acid residues, for example fragment 17--41 with antibacterial activity. A fragment characterised by antibacterial activity was also identified in β-lactoglobulin. Ouwehand and Salminen [1998] have demonstrated that β-lactoglobulin isolated from different types of milk may possess nonspecific antibacterial activity in infant nutrition, manifested by its varied ability to inhibit the adhesion of *E. coli* to the intestinal epithelium.

Lactoferrin is the only milk protein which can be a source of peptides with anticarcinogenic activity. The above is demonstrated by the presence of two fragments with

FKCRRWQWRMKKLGAPSITCVRRAF and RRWQWR sequences located at positions 17-41 and 20-25 [Vogle et al. 2002, Wakabayashi et al. 1996].

Table 7 lists enzymes releasing *in silico* bioactive peptides from milk proteins. The resulting fragments are mainly peptides with the following types of activity: antihypertensive, opioid, immunomodulating, antithrombotic, regulating ion flow, antiamnestic

Table 7. Enzymes releasing *in silico* bioactive peptides from milk proteins

Activity	Proteolytic enzymes
Antihypertensive	Proteinase K (EC.3.4.21.14), Pancreatic elastase (EC 3.4.21.36), Chymotrypsin A (EC 3.4.21.1), Trypsin (EC 3.4.21.4), Pepsin (EC 3.4.23.1), Prolyl oligopeptidase (EC 3.4.21.26), Thermolysin (EC 3.4.24.27), Chymotrypsin C (EC 3.4.21.2), Plasmin (EC 3.4.21.7), Cathepsin G (EC 3.4.21.20), Papain (EC 3.4.22.2), Metridin (EC 3.4.21.3), Pancreatic elastase II (EC 3.4.21.71), Ficin (EC 3.4.22.3), Bromelain (EC 3.4.22.4), Oligopeptidase B (EC 3.4.21.83), Glycyl endopeptidase (EC 3.4.22.25)
Antioxidative	Chymotrypsin A (EC 3.4.21.1), Pepsin (EC 3.4.23.1), Proteinase K (EC.3.4.21.14), Pancreatic elastase (EC 3.4.21.36), Thermolysin (EC 3.4.24.27), Cathepsin G (EC 3.4.21.20), Metridin (EC 3.4.21.3), Pancreatic elastase II (EC 3.4.21.71), Papain (EC 3.4.22.2), Ficin (EC 3.4.22.3), Chymotrypsin C (EC 3.4.21.2)
Immunomodulating	Pancreatic elastase (EC 3.4.21.36), Glycyl endopeptidase (EC 3.4.22.25), Chymase (EC 3.4.21.39)
Antithrombotic, antiamnestic, regulating stomach mucosal membrane activity	Proteinase K (EC.3.4.21.14), Pancreatic elastase (EC 3.4.21.36), Prolyl oligopeptidase (EC 3.4.21.26), Chymotrypsin C (EC 3.4.21.2), Trypsin (EC 3.4.21.4), Plasmin (EC 3.4.21.7), Cathepsin G (EC 3.4.21.20), Oligopeptidase B (EC 3.4.21.83), Papain (EC 3.4.22.2), Ficin (EC 3.4.22.3), Bromelain (EC 3.4.22.4), Glycyl endopeptidase (EC 3.4.22.25)
Smooth muscle contracting	Trypsin (EC 3.4.21.4), Plasmin (EC 3.4.21.7), Oligopeptidase B (EC 3.4.21.83)
Opioid	Pancreatic elastase (EC 3.4.21.36), Glycyl endopeptidase (EC 3.4.22.25), Chymase (EC 3.4.21.39)
Dipeptydyl-peptidase IV inhibitory	Proteinase K (EC.3.4.21.14), Pancreatic elastase (EC 3.4.21.36), Prolyl oligopeptidase (EC 3.4.21.26), Thermolysin (EC 3.4.24.27), Chymotrypsin C (EC 3.4.21.2), Papain (EC 3.4.22.2), Ficin (EC 3.4.22.3), Leukocyte elastase (EC 3.4.21.37), Bromelain (EC 3.4.22.4)
Regulating phosphoinositole mechanism	Chymase (EC 3.4.21.39)
Opioid antagonist	Trypsin (EC 3.4.21.4), Plasmin (EC 3.4.21.7), Oligopeptidase B (EC 3.4.21.83), Pancreatic elastase (EC 3.4.21.36),
Antibacterial and antiviral	Trypsin (EC 3.4.21.4), Plasmin (EC 3.4.21.7), Bromelain (EC 3.4.22.4), Oligopeptidase B (EC 3.4.21.83)

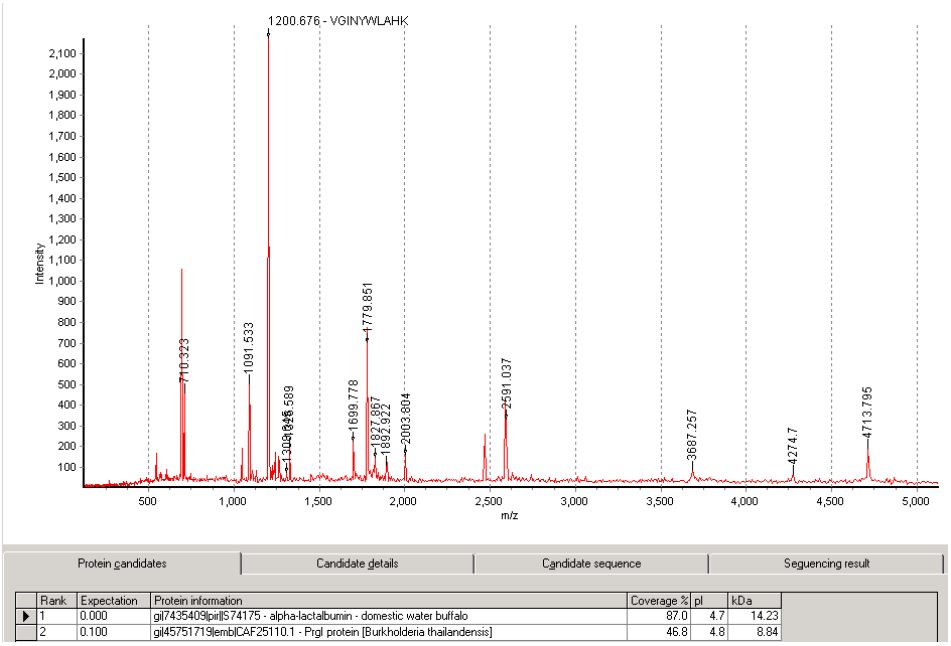

Fig. 1. Mass spectrum of a tryptic hydrolysate of cow's α-lactalbumin

and dipeptidyl peptidase IV inhibiting. Enzymes specific for milk proteins which produce the highest quantity of the above peptides are elastase, chymotrypsin and trypsin. Peptides released from all milk proteins are mainly dipeptides and tripeptides, e.g. RL, GY, AY, VPL, QP, LW, FY, FP, YL, FGK, RF, AP. These short peptides are most readily absorbed from the gastrointestinal tract into the bloodstream [Siemensma et al. 1993, Dziuba et al. 2002].

According to research results, peptides which are suitable for use as physiologically active food components, i.e. peptides marked by antihypertensive activity, peptides responsible for ion metal transport, peptides with immunomodulating, antibacterial and antioxidative activity, are generally not hydrolysed by proteolytic enzymes of the digestive tract. A comparison of the specificity of digestive enzymes with the sequence of physiologically important peptides confirms that they are generally resistant to proteolysis. Peptides containing proline (P) inside a sequence motif are an exception. Those peptides are hydrolysed by proline oligopeptidase. Therefore, designers of bioactive peptide ingredients should first investigate whether intermolecular peptide bonds are hydrolysed by digestive enzymes.

The results of *in silico* proteolysis suggest that trypsin may release many active fragments from precursor proteins. This enzyme is also capable of releasing peptides with antiviral activity from lactoferrin and peptides with antihypertensive activity from α-lactalbumin, κ-casein and β-casein. The mass spectrum of α-lactalbumin trypsin hydrolysate in Figure 1 showed a peak with MW of 1200 Da. This peak corresponded to fragment 98-107 of α-lactalbumin with sequence VGINYWLAHK. This peptide demonstrates antihypertensive activity.

CONCLUSIONS

Based on an analysis of the potential activity profiles and the occurrence frequency of bioactive motifs in milk protein structure, it can be concluded that those proteins are a source of peptides with mainly the following types of activity: antihypertensive, immunomodulating, smooth muscle contracting, antioxidative, dipeptidyl peptidase IV inhibiting, opioid, opioid antagonistic, bonding and transporting metals, antibacterial, antiviral and antithrombotic.

Enzymes specific for milk proteins which produce the highest quantity of the above peptides are elastase, chymotrypsin and trypsin. Peptides released from milk proteins by those enzymes are mainly dipeptides and tripeptides, e.g. RL, GY, AY, VPL, QP, LW, FY, FP, YL, FGK, RF, AP. According to research results, peptides which are suitable for use as physiologically active food components, i.e. peptides marked by antihypertensive activity, peptides responsible for ion metal transport, peptides with immunomodulating, antibacterial and antioxidative activity, are generally not hydrolysed by proteolytic enzymes of the digestive tract.

The results of a computer-aided simulation of protein proteolysis, verified experimentally for lactoferrin, α-lactalbumin, β-casein and κ-casein hydrolysed by trypsin, indicate that they are a relatively abundant source of biologically active peptides. The phrase "relatively abundant source" relates to the comparison of the results of the computerised simulation of the proteolysis process with experimental results rather than to the general number of bioactive motifs in precursor proteins. Milk proteins are a highly abundant potential source of bioactive peptides. The released peptides may be used as diet supplements, natural preservatives and nutraceutics. The relevant research poses a significant challenge and it may contribute to the development of products that have a beneficial effect on human health.

REFERENCES

Bairoch A., Apweiler R., 2000. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 28(1), 45-48.

Bennett K.L., Brønd J.C., Kristensen D.B., Podtelejnikov A.V., Wiśniewski J.R., 2004. Analysis of large-scale MS data sets: the dramas and the delights. DDT: Targets 3(2) (Suppl.), 43-49.

Bray J.E., Todd A.E., Pearl F.M., Thornton J.M., Orengo C.A., 2000. The CATH dictionary of homologous superfamilies (DHS): a consensus approach for identifying distant structural homologues. Protein Eng. 13(3), 153-165.

Bush R.K., Hefle S.L., 1996. Food allergens. Crit. Rev. Food Sci. Nutr. 36, 119-163.

Clark D.A., Swaisgood H.E., 2000. Bioactive milk peptides: A prospectus. J. Dairy Sci. 83, 1187--1195.

Colinge J., Masselot A., 2004. Mass spectrometry has married statistics: uncle is functionality, children are selectivity and sensitivity. DDT: Targets 3(2) (Suppl.), 50-55.

Coste M., Rochet V., Léonil J., Mollé D., Bouhallab S., Tomé D., 1992. Identification of C-terminal peptides of bovine β-casein that enhance proliferation of rat lymphocytes. Immunol. Lett. 33, 41-46.

Dziuba J., Iwaniak A., Darewicz M., Niklewicz M., 2002. Computer simulation of animal proteins proteolysis in the aspect of bioactive peptides obtaining. Pol. J. Food Nutr. Sci. 10(1), 209-222.

Dziuba J., Minkiewicz P., Nałęcz D, Iwaniak A., 1999 a. Database of biologically active peptide sequences. Nahrung 43(3), 190-195.

Dziuba J., Minkiewicz P., Nałęcz D., 1999 b. Biologically active peptides from plant and animal proteins. Pol. J. Food Nutr. Sci. 8/49 (1), 3-16.

Friedman M., 1996. Nutritional value of proteins from different food sources. A review. J. Agric. Food. Chem. 44, 6-29.

Fukudome S., Yoshikawa M., 1992. Opioid peptides from wheat gluten: their isolation and characterisation. FEBS Lett. 296, 107-111.

Gobbetti M., Ferranti P., Smacchi E., Gofferdi F., Addeo F., 2000. Production of angiotensin-I--converting-enzyme-inhibitory peptides in fermented milks by *Lactobacillus delbruecki* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. App. Environ. Microbiol. 66(9), 3898-3904.

Gobbetti M., Stepaniak L., De Angelis M., Corsetti A., Di Cagno R., 2002. Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy. Crit. Rev. Food Sci. Nutr. 42(3), 223-239.

Juillard V., Laan H., Kunji R.E.S., Jeronimus-Stratingh C.M., Bruins A.P., Konings W.N., 1995. The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes β-casein into more than one hundred different oligopeptides. J. Bacteriol. 177, 3472-3478.

Karelin A.A., Blishchenko E.Yu., Ivanov V.T., 1998. A novel system of peptidergic regulation. FEBS Lett. 428, 7-12.

Kilara A., Panyam D., 2003. Peptides from milk proteins and their properties. Crit. Rev. Food Sci. Nutr. 43(6), 607-633.

Korhonen H., Pihlanto-Leppälä A., 2001. Milk protein-derived bioactive peptides – novel opportunities for health promotion. Bull. IDF 363, 17-26.

Kriventseva E.V., Fleischmann W., Zdobnov E.M., Apweiler R., 2001. CluStr: a database of clusters of SWISS-PROT + TrEMBL proteins. Nucleic Acids Res. 29(1), 33-36.

Lackner P., Koppensteiner W.A., Dominques F.S., Sippl M.J., 1999. Numerical evaluation methods. Automated large scale evaluation of protein structure prediction. Proteins 3, 7-14.

Meisel H., Bockelmann W., 1999. Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functinal properties. Antonie van Leeuwenhoek 76, 207-215.

Meisel H., 1998. Overview on milk protein-derived peptides. Int. Dairy J. 8, 363-373.

Ouwehand A.C., Salminen S.J., 1998. Adhesion inhibitory activity of β-lactoglobulin isolated from infant formulae. Acta Paedriat. 87, 491-493.

Pereira S.P., Ciclitira P.J., 2004. The pathogenesis of coeliac disease. Int. J. Biochem. Cell Biol. 1, 17-24.

Pihlanto-Leppälä A., 2001. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. Trends Food Sci. Technol. 11, 347-356.

Pihlanto-Leppälä A., Rokka T., Korhonen H., 1998. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. Int. Dairy J. 8, 325-331.

Saito T., Nakakura T., Kitazawa H., Kawai Y., Itoh T., 2000. Isolation and structural analysis of antihypertensive peptides that exist naturally in gouda cheese. J. Dairy Sci. 83, 1434-1440.

Schanbacher F.L., Talhouk R.S., Murray F.A., 1997. Biology and origin of bioactive peptides in milk. Liv. Prod. Sci. 50, 105-123.

Schlimme E., Meisel H., 1995. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. Nahrung 39, 1-20.

Siemensma A.D., Weijer W.F.I., Bak H.J., 1993. The importance of peptide lenghts in hypoallergenic infant formule. Trends Food Sci. Technol. 4, 16-21.

Vogle H.J., Schibli J.D., Jing W., Epand F.R., Epand R.M., 2002. Towards a structure-function analysis of bovine lactoferricin and related tryptophan and arginine containing peptides. Biochem. Cell Biol. 80, 49-63.

Wakabayashi H., Hiratani T., Uchida K., Yamaguchi H., 1996. Antifungal spectrum and fungicidal mechanism of an N-terminal peptide of bovine lactoferrin. J. Infect. Chemother. 1, 185-189.

BIAŁKA MLEKA JAKO PREKURSORY PEPTYDÓW BIOAKTYWNYCH

Streszczenie. Białka mleka jako źródło peptydów bioaktywnych są przedmiotem wielu badań naukowych. Problematyka jest związana między innymi z oceną tych białek jako prekursorów peptydów biologicznie aktywnych. Uwolnione ze swoich prekursorów fizjologicznie aktywne peptydy mogą oddziaływać z określonymi receptorami i wpływać na ogólną kondycję i zdrowie człowieka. Na podstawie utworzonej w Katedrze Biochemii Żywności UWM w Olsztynie bazy białek i bioaktywnych peptydów – BIOPEP (www.uwm.edu.pl/biochemia) wyznaczono profile potencjalnej aktywności białek mleka oraz przeprowadzono ocenę wartości tych białek jako prekursorów peptydów bioaktywnych z wykorzystaniem kryterium ilościowego, tj. częstości występowania fragmentów bioaktywnych (A). Wykazano, że białka mleka mogą być głównie źródłem peptydów o aktywności przeciwnadciśnieniowej ($A_{maks.}$ = 0,225), immunomodulacyjnej (0,024), wywołującej skurcze mięśni gładkich (0,011), przeciwutleniającej (0,029), inhibitora dipeptydylopeptydazy IV (0,148), opioidowej (0,073), antagonistycznej w stosunku do opioidowej (0,053), wiązania i transportowania metali i jonów metali (0,024), antybakteryjnej i antywirusowej (0,024) oraz przeciwkrzepliwej (0,029). Dla wszystkich aktywności ustalono, które enzymy mogą uwalniać bioaktywne peptydy z białek prekursorowych. Ponadto potwierdzono eksperymentalnie, że istnieje relatywnie duża możliwość otrzymywania peptydów aktywnych biologicznie, po hydrolizie trypsyną, takich białek mleka, jak laktoferyna, laktoalbumina α, kazeina κ i β.

Słowa kluczowe: peptydy bioaktywne, białka mleka, proteoliza *in silico*

Accepted for print – Zaakceptowano do druku: 5.12.2008

For citation – Do cytowania: Dziuba M., Dziuba B., Iwaniak A., 2009. Milk proteins as precursors of bioactive peptides. Acta Sci. Pol., Technol. Aliment. 8(1), 71-90.