Identification of bioactive peptides from camel milk whey protein with potential health benefits through control of oxidative stress, glucose release after starch digestion, and pathogen adhesion

by

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Abstract

Camel milk cheese is popular in many countries and the production of camel milk cheese has increased, as has its by-product whey. The prevalence of non-communicable diseases, mainly diet-related chronic diseases like diabetes mellitus (DM), has increased worldwide. On other hand, the enterotoxigenic *Escherichia coli* (ETEC) is a major cause of childhood diarrhea and diarrhea in piglets and calves. This research aimed to investigate the potential antioxidant activities, starch digestion inhibitory activities, and antiadhesive activity against ETEC of bioactive peptides from camel milk whey protein (CMWP). CMWP was hydrolyzed by flavourzyme, neutrase, alcalase, or a mixture of neutrase and flavourzyme. The antioxidant activities of the hydrolysates were determined using 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferrous ion (Fe^{2+}) chelating activity, superoxide ($O^{\cdot 2-}$) free radical scavenging, and measurement of reducing power. The antioxidant activity of flavourzyme hydrolysates (FH) at 1.5 h hydrolysis was highest and pepsin digested FH effectively to obtain peptides with high antioxidant activity, whereas trypsin digestion negatively affected antioxidant activities. The peptic hydrolysates were characterized by one sharp main peak of molecular weight (MW (6.83KDa)); however, further hydrolysis by trypsin led to hydrolysis of the peptides to amino acids and very short peptides. The low-medium (2KDa < size < 10KDa) sized peptides exhibited the highest antioxidant activities. Starch digestion inhibitory activity of bioactive peptides derived from CMWP was investigated using a digestibility assay that included addition of pepsin, pancreatic enzymes, and brush border enzymes. The hydrolysates from the flavourzyme hydrolysis were fractionated either by hydrophobic interaction chromatography (HIC), or by cation exchange chromatography (CEX) followed by HIC. The successive chromatographic separation aiming to produce positively charged peptide with hydrophobic amino acids improved the starch digestion inhibition. Fractions

that inhibited starch digestion the most were selected for peptide sequencing by LC-MS/MS. Among the sequenced peptides, six short peptides were chosen for peptide synthesis. The original unfractionated hydrolyzed whey showed about 16.5% inhibition of starch digestion. However, the fractionation on HIC column alone or on CEX and HIC boosted the inhibitory activity by about 63% and 116%, respectively. LALDIEIATYR and VLDELTLAR had the same activities as the entire fraction. Sweet whey from camel milk contains caseinomacropeptide (CMP) and glycomacropeptide (GMP). The GMP from camel milk was purified by ion exchange chromatography and ultra-filtration, and the purity of camel GMP determined by SDS-PAGE and mass spectrometry. The anti-adhesion activity of camel GMP against ETEC was determined using an hemagglutination assay and by enzyme-linked immunosorbent assay (ELISA). The monosaccharide content of GMP from Bactrian camels and dromedaries was about twice as high when compared to bovine GMP. Glycans from camels included fucose and N-acetylglucosamine, which were absent in bovine GMP. GMP from both camel's species prevented ETEC adhesion to porcine blood cells at a concentration of 0.24 g/L and 0.28 g/L respectively, a concentration that is about 20-fold lower when compared to bovine. This increased activity likely relates to the increased glycosylation and the density of glycan spacing, and / or to differences in the glycan composition. In conclusion, bioactive peptides derived from CMWP have potential inhibitory effect on oxidative stress, starch digestion, and pathogenic bacteria adhesion. .

Preface

This thesis is the original work by Rami Althnaibat.

Chapter 2 is a literature review being prepared as a manuscript for publication as Rami Althnaibat, Heather Bruce, Jianping Wu, and Michael Gänzle "Potential applications of bovine and camel milk proteins hydrolysates for controlling hyperglycemia, blood hypertension, and pathogen adhesion: A review of randomized clinical studies", Dr. Gänzle was the corresponding author contributed to manuscript composition, editing, and revision. Dr. Wu contributed to revision.

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Dedication

To my parents

To Dr. Heather Bruce and Dr. Michael Gänzle

To my wife and my daughters

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List of Abbreviation

ACE	Angiotensin converting enzyme
AH	Alcalase hydrolysates
Ala (A)	Alanine
ANS	8-anilino-1 naphthalene sulfonic acid
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
BPs	Bioactive peptides
BSA	Bovine serum albumin
CEX	Cation exchange chromatography
CHO	Carbohydrate
CM	Camel milk
C_{max}	Maximum concentrations
CMWP	Camel milk whey proteins
Cys (C)	Cysteine
DBP	Diastolic blood pressure
DH	Degree of hydrolysis
DM	Diabetes mellitus
DP	Degree of polymerization
DPP4	Dipeptidyl peptidase-4
DPPH	1,1- diphenyl-2-picrylhydrazyl
EHEC	enterohemorrhagic *E. coli*

ELISA	Enzyme linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
F	Fraction
FAO	Food and Agriculture Organization of the United Nations
FH	Flavourzyme hydrolysates
Gal	Galactose
GI tract	Gastrointestinal tract
GIP	Glucose-dependent insulinotropic polypeptide
Glc	Glucose
GlcN	N-glucosamine
GlcNAc	N-acetyl glucosamine
Gln (Q)	Glutamine
GLP	Glucagon like-peptide-1
Glu (E)	Glutamic acid
Gly (G)	Glycine
GMP	Glycomacropeptide
GOS	Galacto oligosaccharides
HCL	Hydrogen chloride
HIC	Hydrophobic interaction chromatography
His (H)	Histidine
HLT	Heat labile enterotoxin
HMOs	Human milk oligosaccharides
HPLC	High-performance liquid chromatography

Ig	Immunoglobulin
Ile (I)	Isoleucine
LC-MS/MS	Liquid chromatography–mass spectrometry
Leu (L)	Leucine
LF	Lactoferrin
Lys (K)	Lysine
MAC	Minimum anti-adhesive concentration
Met (M)	Methionine
MIC	Minimum inhibitory concentration
Mw	Molecular weight
NeuNAc	N-acetylneuraminic acid
O^{2-}	Superoxide radical
OD	Optical density
PBS	Phosphate buffer solution
Phe (F)	Phenylalanine
pI	Isoelectric point
Pro (O)	proline
PYY	Peptide tyrosine-tyrosine
RCT	Randomized clinical trials
RDS	Rapidly digestible starch
ROS	Reactive oxygen species
RS	Resistant starch
SBP	Systolic blood pressure

SDS	Slowly digestible starch
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SE-HPLC	Size exclusion-high performance liquid chromatography
Ser (S)	Serine
ST	Heat stable enterotoxin
STEC	Shiga toxin-producing *E. coli*
$t_{1/2}$	The elimination half-lives
T2D	Type2- diabetes
TCA	Trichloroacetic acid
Thr (T)	Threonine
TNBS	Trinitro benzene sulfonic acid
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine
α-LA	α-lactalbumin
β-lg	β-lactoglobulin

Chapter 1. **Introduction**

1.1. Camel overview

Camel (*Camelus*) is the sixth domesticated animals on earth and the fifth major dairy animal after cow, sheep, goat, and buffalo by population [1]. The camel is related to the family Camelidae, and can be divided into three genera; *Camelus*, *Lama*, and *Vicugna* (Figure 1.1) [1]. Camels from the genus Camelus can be further classified into two main species based on the number of humps. The one-humped camels (*Camelus dromedaries*), also known as Arabian camel or dromedary, are usually found in hot regions, such as Africa and the Middle East. The two-humped (*Camelus bactrianus*) camels, known as Bactrian camels, live in cold areas like southern Russia and central China [2]. In addition to that there are four other types of small camelids, Llama, Alpaca, Guanaco, and Vicuna, which are found in the Andin Mountain region of the South America. These camels are called the "South American Camelidae" and the first two types of them belong to tame camelids and are called the "New World camels", whereas the other two types are untamed camelids and are called the "South American camels" [1,3].

The exact number of camel populations in the world is unknown because camels are mainly used by nomadic and pastoralist areas people [4,5]. However, the Food and Agriculture Organization of the United Nations (FAO) has reported in 2022 an estimation of about 39 million camels (*Camelus*) in the world [6].

1.2. Camel milk

Milk is one of the main foods that contain a great number of essential and bioactive molecules like lipids, vitamins, minerals, and proteins with diverse therapeutic activities [7]. Milk is commercially

produced from many dairy animals such as cows, goats, sheep, and camels. Camels are a good source of meat and milk.

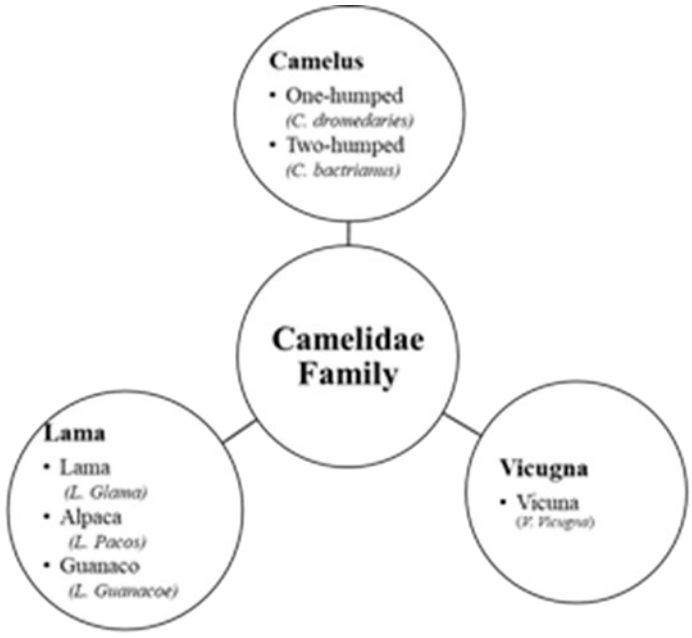


Figure 1.1: Camel family classification [1].

The climate of the camel's habitat [8], seasonal variations [5], and the breed [7], have a direct impact on the solids content of the camel milk (Table 1.1). Camel milk has a distinctive sweet flavour and is slightly salty in taste [9]. The color of camel milk is bleary white which is due to the presence of a trace amount of vitamin A (carotene) [10]. Camel milk contains high concentrations of water-soluble vitamins, mainly vitamin C and some vitamin B-complex. It has been reported that camel milk has about 34.16 mg/L of vitamin C, which is three times more than that in cow milk [9].

Table 1.1: Chemical and molecular composition of camel, cow, goat, and sheep milk

	Average content%					Number of Amino Acids				
	Dromedary	Bactrian	Cow	Goat	Sheep	Dromedary	Bactrian	Cow	Goat	Sheep
Water%	88	86	87	88	81	---	---	---	---	---
Total solid%	12	14	13	12	19	---	---	---	---	---
Fat%	3.6	5.3	3.6	3.8	6.9	---	---	---	---	---
Fat globule size (µm)	3.0	3.0	4.3	3.2	3.6	---	---	---	---	---
Lactose%	4.6	4.5	4.9	4.1	4.8	---	---	---	---	---
Ash%	0.8	0.8	0.7	0.8	1.0	---	---	---	---	---
Protein%	3.3	3.9	3.3	3.5	5.0	---	---	---	---	---
Casein %	2.5	2.9	2.5	2.3	4.1	---	---	---	---	---
Casein micelle size(µm)	0.4	0.4	0.2	0.3	0.2	---	---	---	---	---
α -casein% [a]	32	32	48	25	30	---	---	---	---	---
α s1 -casein% [a]	22	22	38	5.6	6.7	207	207	199	199	199
α s2-casein% [a]	9.5	9.5	10	19	23	178	178	207	208	208
β -casein %[a]	65	65	39	55	62	217	217	209	207	207
κ -casein %[a]	3.5	3.5	13	20	8.9	162	162	169	171	171
Whey proteins%	0.8	1.0	0.8	1	0.9	---	---	---	---	---
α-lactalbumin (α-LA) %[b]	47	47	20	27	15	123	123	123	123	123
Immunoglobulin (Ig)% [b]	7.8	7.8	13	9.7	7.3	---	---	---	---	---
β-lactoglobulin (β-LG) % [b]	----	----	59	59	73	---	---	162	162	162
Lactoferrin (LF)% [b]	5.4	5.4	1.8	0.1	0.1	689	689	689	689	689
Serum albumin %[b]	9.43	9.43	6.2	4.0	4.1	588	588	583	583	583
Lysozyme mg/L[b]	38	38	18	trace	trace	---	---	---	---	----

[a]; % from total casein content, [b]; % from total whey protein content. The data in the table obtained from [10, 11, 3, 12, 5, 13, 14, 15, 16, 17, 18, 19].

Total milk production varies from 320 to 3800 liters per year based on the age of the camels from 8-18 months. Moreover, the milk production per animal varies by the camel breed; for example, a dromedary camel produces 6-7 L of milk per day, whereas a Bactrian camel produces 0.5-1 L/day [20]. The properties of camel milk are also determined by the age of the animal, their living conditions (climate) and geographical locations, type of fodder, frequency of pregnancy, and the

manner and time of lactation [7]. The protein content in camel milk is high and is the third major constituent of the milk and comprises an average of 3.3% and 3.9% of camel milk solids from Dromedary and Bactrian types, respectively [10]. Camel milk protein contains a high proportion of essential amino acids. Camel milk has many other special properties like the presence of α-lactalbumin protein, the absence of β-lactoglobulin protein, and a different ratio between casein and whey protein compared to cow, sheep, and goat milk [21].

Like other milk, camel milk has casein as the most dominant protein. Casein can be divided into three fractions: β-casein, κ-casein, and α-casein with proportions of 65%, 3.5%, and 31.5%, respectively. The α-casein can be further divided into two types, αs1-casein, and αs2-casein, with proportions of 22% and 9.5% of the total casein, respectively [22]. The size of the casein micelle in camel milk is larger than the casein micelle of other species. The diameter of casein micelle is about 380 nm in camel milk, and 150, 260, and 180 nm, for cow, goat, and sheep milk, respectively [16] (Table 1.1). The casein protein of camel milk is susceptible to pH (the isoelectric point, pI of camel milk at pH 4.3) [23]. The amino acid compositions of casein from cow milk and camel milk are very similar except casein from camel milk has low concentrations of glycine and cysteine [19].

κ-Casein

κ-Casein is the smallest fraction of the camel milk casein and constitutes about 3.5% of the total casein protein. On the other hand, the κ-casein in cow milk represents 13% of the total casein and contains higher concentrations of essential amino acids except for lysine compared to camel milk κ-casein [24,25]. Moreover, the camel milk κ-casein amino acid sequence has an additional proline residue (pro95) that contributes to camel milk's better heat stability than the cow, goat, and sheep milk [19].

The κ-casein from camel milk has a molecular weight of 22.29- 22.99 kDa. The κ-casein of cow, goat and sheep milk can be hydrolyzed by chymosin (Rennet) at Phe105-Met106 during cheese processing, whereas the camel milk κ-casein is hydrolyzed by rennet Phe97-Ile98 [19,26] (Table 1.2). The caseinomacropeptide (CMP; not glycosylated) and glycomacropeptide (GMP; glycosylated) representing the C-terminal of κ-casein of camel milk can be obtained by rennet hydrolysis of the milk protein. This hydrolysis causes the separation of casein from whey protein during the making the cheese. CMP is the third most abundant bioactive compound in cheese whey protein, constituting about 15-20% of the total whey proteins. Hydrolysis of cow milk κ-casein at Phe105-Met106 linkage releases a polar and non-polar polypeptide. The non-polar peptide is known as para κ-casein, consists of 105 amino acids, and remains in the curd [27,25]. The highly polar peptide consists of 64 amino acids (Met-106 - Val-169 residue) and remains in the whey protein (liquid phase) [27,28].

Table 1.2: κ -Casein residue sequences for (camel, cow, goat, and sheep), and their chymosin's cleavage site. Green-colored boxes indicate the cleavage site of rennet [19,29,30,31].

Animal	κ -casein residue sequence
Camel	EVQNQEQPTC FEKVERLLNE KTVKYFPIQF VQSRYPSYGI NYYQHRLAVP 50 INNQFIPYPN YAKPVAIRLH AQIPQCQALP NIDPPTVERR PRPRP$FIAI 100 PPKKTQDKTV NPAINTVATV EPPVIPTAEP AVNTVVIAEA SSEFITTSTP 150 ETTTVQITST EI 162
Cow	QEQNQEQPIR CEKDERFFSD KIAKYIPIQY VLSRYPSYGI NYYQQKPVAL 50 INNQFLPYPY YAKPAAVRSP AQILQWQVLS NTVPAKSCQA QPTTMARHPH 100 PHLSFMAIPP KKNQDKTEIP TINTIASGEP TSTPTTEAVE STVATLEDSP 150 EVIESPPEIN TVQVTSTAV 169

Goat	QEQNQEQPIC CEKDERFFDD KIAKYIPIQY VLSRYPSYGL NYYQQRPVAL 50 INNQFLPYPY YAKPVAVRSP AQTLQWQVLP NTVPAKSCQD QPTTLARHPH 100 PHLSFMAIPP KKDQDKTEVP AINTIASAEP TVHSTPTTEA IVNTVDNPEA 150 SSESIASASE TNTAQVTSTE V 171
Sheep	QEQNQEQRIC CEKDERFFDD KIAKYIPIQY VLSRYPSYGL NYYQQRPVAL 50 INNQFLPYPY YAKPVAVRSP AQTLQWQVLP NAVPAKSCQD QPTAMARHPH 100 PHLSFMAIPP KKDQDKTEIP AINTIASAEP TVHSTPTTEA VVNAVDNPEA 150 SSESIASAPE TNTAQVTSTE V 171

Whey protein

Whey protein is the second main protein in camel milk and constitutes about 20-30% of total camel milk proteins [32]. When separated from camel milk, the whey protein is white which is due to the light reflection from the small fat droplets and κ-casein particles. On the other hand, the whey proteins extracted from cow, goat, and sheep milk exhibit a greenish shade[33]. There are many differences between the whey protein of camel milk and cow milk with respect to the thermal stability and acidity. The whey protein from camel milk is stable and less susceptible to heat than cow milk whey protein. This higher thermal stability could be due to the absence of β-lactoglobulin and the low concentration of κ-casein in camel milk[34]. The major characteristics of whey proteins from camel milk are highlighted in Table 1.1.

Whey protein from camel milk contains high amounts of α-lactalbumin, lactoferrin, antibodies and immunologically active ingredients like serum albumin, peptidoglycan, lysozyme, immunoglobulin, and lactoperoxidase. Camel milk whey proteins have a suitable balance between essential and non-essential amino acids [35]. The α-lactalbumin (α-LA) is the major component of whey protein in camel milk and comprises about 47.41 % of the total whey proteins in camel milk, whereas, in cow, goat, and sheep milk α-LA contents are about 20.1, 27, and 14.8% of total whey

proteins (Table 1.1). The high concentration of α-LA in camel milk whey protein affects the solubility of protein and makes the whey protein more susceptible to the pH [34].

The β-lactoglobulin (β-LG) is the major whey protein in cow milk and constitutes about 58% of total cow whey proteins, whereas this protein is absent in camel milk whey protein[36]. The content of lactoferrin, immunoglobulins, and serum albumin in camel milk is around 5.4%, 7-8%, and 9.4 of the total whey protein, respectively [37]. The lysozyme concentrations in camel milk are about 228-500 μg/100 mL, and are highest in colostrum [38]. The lysozyme concentration in camel milk is about 11, 8, and 10 times higher than that in cow, goat, and sheep milk, respectively [39].

Amino acid composition

In general, whey and casein proteins have an equilibrium of essential and non-essential amino acids, but the concentration varies by type of the milk [40]. A study by Rafiq and others (2016) of the amino acid compositions for camel, cow, goat, sheep, and buffalo milk reported that leucine and valine are present in the highest concentrations in cow milk followed by camel milk[41]. Camel milk whey proteins contain most of the essential amino acids (i.e., Phe, Val, Thr, Try, Met, Leu, Ile, Lys, and His) with high concentrations of Phe, Val, Leu, and Lys. On the other hand, the non-essential amino acids of camel milk whey protein are present in low quantities except Glu and Pro. These characteristics support the hypothesis that camel milk whey protein is a promising candidate to produce bioactive peptides with health benefits which can be a major part of functional foods [21].

1.3. Camel milk cheese

Cheese processing is usually restricted to goat, cow, and sheep milk. Camel milk is widely consumed in North Africa and in the Middle East [42] and most camel milk is consumed as raw milk

without any processing. Recently, camel milk has attracted attention due to its unique biological and therapeutic properties [43], and many processed products such as cheese, chocolate, butter, ice cream, yogurt, and fermented milk made from camel milk have been developed and are available in the market [44,45]. The production of many different types of camel cheese such as fresh, soft, and semi-hard has increased and thus increased the amount of whey as a by-product [42,46]. The proportion of casein and whey proteins in camel milk is different than that in milk from other species. The ratio of casein and whey proteins in bovine, ovine, and caprine milk is (~80:20) of the total milk protein, whereas, this ratio in camel milk is about 75:25 [21]. Until now in developing countries, whey has been considered a waste product that requires expensive treatment before discharging into the environment. In some cases, whey is processed into relatively low-value commodities such as whey powder or whey protein concentrates for use as a food ingredient. Therefore, the full potential of whey has not yet been explored [47].

Whey contains a rich mixture of soluble proteins with different chemical, physical and functional properties. Whey proteins from cow milk or their derivatives have potential health benefits [48]. Specific use of these proteins in biologically functional foods, nutraceuticals, pharma-foods, or designer foods could then potentially improve human and animal health [48]. Most of the research on whey protein is focused on that sourced from bovine milk, while a few studies have focused on that derived from camel milk.

1.4. Hypotheses

The hypotheses examined in this thesis, therefore, are:

- *In vitro* enzymatic hydrolysis of whey proteins from camel milk in a controlled manner produce beneficial bioactive peptides with antioxidant activity.

- Hydrolysis of whey proteins from camel milk using fungal and intestinal enzymes releases peptides that inhibit starch digestion.

- Chymosin hydrolysis, ultrafiltration, and ion exchange chromatography produces pure glycomacropeptide from camel milk.

- GMP derived from camel milk has anti-adhesive effects against Enterotoxigenic *Escherichia coli* K88.

1.5. Objectives

The overall objective of this thesis is to investigate the effects of enzymatic hydrolysis and successive chromatographic fractionation on camel milk whey protein to produce bioactive peptides and glycopeptides, and to determine antioxidant, starch digestion inhibition, and anti-adhesion activities for produced peptides. The overall objective can be divided into four specific objectives:

Objective 1: to characterize the *in vitro* antioxidant activities of the bioactive peptides derived from camel milk whey protein produced using enzymatic hydrolysis.

Objective 2: to determine the effect of peptides from camel milk whey protein on the inhibition of the starch digestion and the effect of positively charged hydrophobic amino acids content on starch digestion inhibitory activity.

Objective 3: to optimize the isolation and purification process of GMP from camel milk whey protein and evaluate its chemical composition.

Objective 4: to investigate the effect of GMP on adhesion of enterotoxigenic *Escherichia coli* K88 to porcine blood cells.

Chapter 2. **Potential applications of bovine and camel milk proteins hydrolysates for controlling oxidative stress, hyperglycemia, blood hypertension, and pathogen adhesion: A review of randomized clinical studies**

-Advanced revision of this chapter has been prepared as a literature review manuscript for publication.

2.1. Introduction

Dietary protein and derived bioactive peptides (BPs) have health-beneficial properties[49]. Bioactive peptides can be derived from dietary proteins by chemical or enzymatic hydrolysis through protein hydrolysis during food processing or food fermentation, and / or during intestinal transit [3]. The health promoting properties of bioactive peptides depend on the molecular weight and the peptide sequence, which determines charge and hydrophobicity [3]. Pepsin hydrolysis in the stomach is the first step of food protein digestion; proteins are then further hydrolyzed by the pancreatic proteases trypsin and chymotrypsin, and by brush border peptidases that are expressed in the mucosal membrane of the small intestine [50]. Bioactive peptides derived from milk are generated by hydrolysis of proteins *in vivo* and/or *ex vivo* through digestive enzymes, microbial enzymes, and microbial fermentation [3]; for example, the casein-derived bioactive peptides VPP and IPP have been detected in the blood stream after consumption of yogurt [51].

Some bioactive peptides act locally in the GIT, examples particularly include peptides that inhibit starch digestion; however, most bioactive peptides including antihypertensive peptides are active after transfer to the blood stream [51]. The bioavailability of peptides is affected by digestive enzymes in the gastrointestinal tract, metabolism, absorption, and distribution or degradation in the blood stream. The bioavailability of ingested bioactive peptides depends on the composition,

number, and sequences of amino acids [51,52,53]. These characteristics of peptides determine the pathway that may be used to cross the intestinal epithelial cell [51].

The bioavailability of bioactive peptides and their transfer to the bloodstream is a major hurdle to health beneficial effects in humans and animals [54]. Hydrolysis of peptides occurs during digestion but also after being transported into the epithelial cells and in the bloodstream [54]. Proteins with high content of proline are resistant to gastric and pancreatic peptidases, and proline-rich peptides are thus most likely to escape the digestion and to reach the intestinal membrane in relatively intact sequence and face the brush border enzyme [53,55]. The milk-derived bioactive peptides IPP [56], VPP [57], PG [58], IP [59], HLPLP [60] have been detected in the plasma of human and animals. However, the peptide concentrations in the blood serum are typically substantially lower than the concentrations that are required for *in vitro* activity [61]. For example, ACE inhibitory activity of the tripeptide IPP is observed at 10 μmol/L [62,63] while the concentration in blood was reported to be 10,000 times lower, 0.90 ± 0.16 nmol / L [59].

Many studies document a significant and favorable effect of some bioactive peptides in human and animal after oral administration. Even for peptides that were shown to be effective in animal models including pigs and rats and in randomized clinical trials (RCT) in humans, the role of bioavailability, effects, pharmacokinetics, and plasma concentrations of bioactive peptides are still not fully understood [51,61]. The maximum concentrations (C_{max}) and the elimination half-lives ($t_{1/2}$) of absorbed bioactive peptides in the blood plasma reflect their bioavailability, and then the possibility of activity [64,65]. Most of the bioactive peptides have achieved their C_{max} in the micromolar range (μM), and $t_{1/2}$ ranged between a few minutes to a few hours. The variation in C_{max} and $t_{1/2}$ of the bioactive peptides in the human plasma could be determined by sex, age,

diseases, interaction with food matrix, in addition to the factors mentioned above that affect their bioavailability [66,67].

Peptide hydrolysis in the small intestinal membrane is mediated by several brush border peptidases. Specifically, brush border enzymes that contribute to peptide hydrolysis include the aminopeptidase N, dipeptidyl aminopeptidase IV, aminopeptidase A, peptidyl dipeptidase, γ-glutamyltranspeptidase, and carboxypeptidase. Of these peptidases, the γ-glutamyltranspeptidase is specifically active on γ-glutamylpeptides of plant or microbial origin. The only enzymes with activity on peptide bonds adjacent to proline are aminopeptidase and carboxypeptidase [50,53,55].

In the last decades, the prevalence of food-related chronic diseases including cardiovascular disease, alcoholic fatty liver disease, diabetes mellitus, and hypertension has increased worldwide [52,68]. Hundreds of *in vitro* studies have suggested that bioactive peptides have a favorable effect on the functions of various organs and that they offer multiple biological and physiological benefits with a wide range of biological activities (Figure 2.1) [69]. Some but not all of these promising *in vitro* data were confirmed *in vivo* with animal models, however, and the susceptibility of orally ingested peptides to GIT and brush border peptidases is a relevant issue to diminish the *in vitro* list of bioactivities for the peptides (Figure 2.1) [70].

Despite promising favorable effects being demonstrated from *in vivo* studies using rodent models, only a limited number of clinical outcomes in humans have been reported. There are many factors that limit randomized clinical trials (RCT) such as insufficient understanding of bioactive peptide's mechanisms of action, inconsistent results, not enough convincing evidence, unclear pharmacokinetics, and methodology limitations [71]. To date, RCT that show a health beneficial effect of dietary bioactive peptides are limited to antihypertensive activity and the

antihyperglycemic effects of peptides that inhibit starch digestion [71,72]. However, antioxidant activities have been demonstrated in *in vitro* studies, while a few *in vivo* studies have demonstrated anti-aging and anti-inflammatory effects related to antioxidant activities for studied peptides [73].

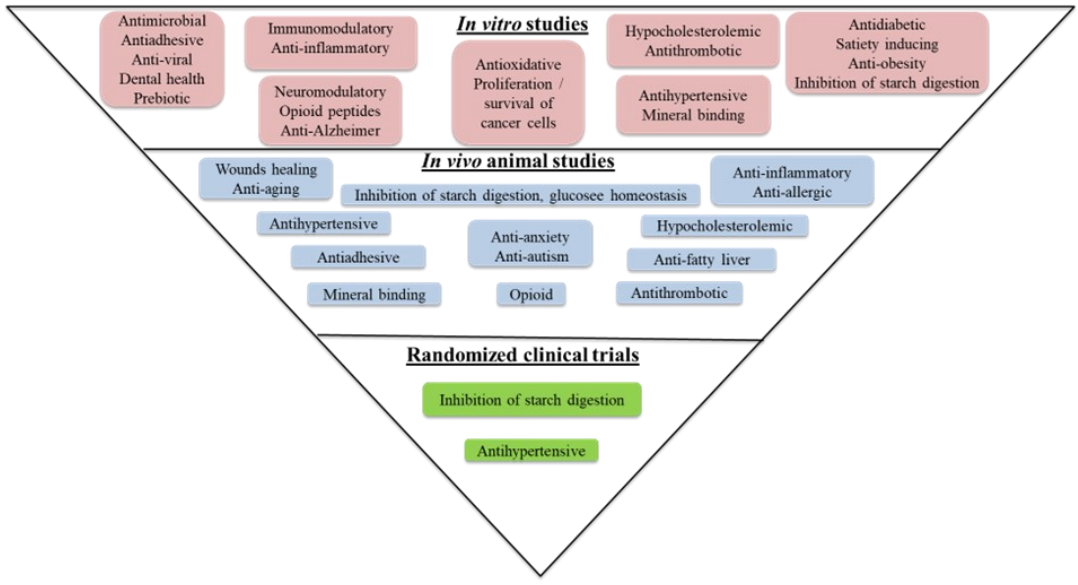


Figure 2.1: In vitro, in vivo, and randomized controlled trails confirmed studies for bioactive peptides

For some peptides, however, animals are not relevant models for human health related issues but are suitable to demonstrate the metabolic relevance of bioactive peptides. This specifically pertains to glycopeptides that aim to prevent adhesion of enterotoxigenic *E. coli* (ETEC) to the intestinal mucosa of swine and calves [74]. These peptides were shown to reduce the cell numbers of ETEC in post-weaning piglets [74,75]. However, the strains of ETEC that cause diarrheal disease in humans differ in the glycan specificity, and successful interventions in swine does not necessarily translate to potential applications in humans [75,76].

Milk is recognized as one of the main dietary sources for health beneficial bioactive compounds. Recently, the research about meat replacement with non-meat protein sources has been increased to reduce overconsumption of meat and facilitate the transition to a sustainable and healthier diet

[77]. Milk products like dairy and cheese represents one of the main non-meat protein foods worldwide. Many of the dairy products are fermented and thus include the fermentation process that releases bioactive peptides by proteolysis. Generally, the fermentation process transforms the lactose to lactic acid, introduce beneficial bacterial microbiota to human gut and thus diminish the lactose intolerance of individuals [78].

Milk-whey is a by-product of the cheese and casein production process, and its volume is on the increase worldwide. Bovine whey contains the whole water of milk with approximately 20% of the original milk protein and therefore represents a significant protein source. As a result, whey is an inexpensive source of high-nutritional quality protein and bioactive peptides for the food and health industries[79]. Sweet whey contains about 5% caseinomacropeptide (CMP), which is an important bioactive peptide with antiadhesion activity against ETEC and many health benefits [74,75]. Until now, whey has been considered a waste product that requires expensive treatment before discharging into the environment in developing countries. In some cases, whey is processed into relatively low-value commodities such as whey protein concentrates to use as food additives to increase the value of whey after remove water and decrease the size of whey. Therefore, the full potential of this resource has not yet been fully explored [47].

Despite the large number of studies on milk-derived bioactive peptides that have been reviewed by many others including Park et al. [3], Nongonierma et al. [56], Horner et al. [65], Cicero et al. [66], Chakrabarti et al. [69], Daroit et al. [70], Duffuler et al. [71], García-Burgos et al. [78], and Luhovyy et al. [79] , most of these studies focus on bovine milk [21]. Only a handful of studies have investigated the bioactive peptides from sheep, goats, horse, or camel milk[21], reflecting that milk from these animals makes only relatively small contribution to the overall liquid milk or fermented dairy product market [80].

Camel milk is widely consumed in many countries of the Middle East and in North Africa [42]. Recently, camel milk attracted attention due to its biological and therapeutic properties [44]. Most of the research on camel milk has focused only on raw milk, casein, and fat. Just a few studies focus on camel milk whey protein [21,44]. In many countries, the production of camel milk cheese has increased; however, this increase in camel milk cheese production also increased the amount of whey as a by-product [42,46]. The proportion of casein and whey proteins in camel milk is different than that in bovine milk. The ratio of casein and whey proteins in bovine milk is about 80%:20% of the total milk protein, meanwhile, this ratio in camel milk is 75%:25% [21].

Therefore, this review aims to summarize the possible bioactivities like antioxidant activities, antihypertension activities, antihyperglycemic activities, and antiadhesion activities against bacteria of peptides derived from bovine and camel milk, with a focus on RCT confirmed studies. Structure-function relationships of antioxidant bioactive peptides will be discussed additionally. Potential health benefits of antioxidant bioactive peptides are documented in multiple in vitro studies meanwhile very limited animal models have documented antioxidant activities that are not confirmed by RCT.

2.2. Antioxidant bioactive peptides

The oxidation reaction is a natural metabolic process in living organisms, but uncontrolled oxidation (imbalance between oxidation and reduction) often produces free radicals or reactive oxygen species (ROS) [81]. The high presence of ROS or free radicals in the body leads to oxidative stress [82]. These highly reactive free radicals steal the donor atom's electrons from the surrounding environment, thereby the donor atoms turn into free radicals and start a chain reaction of oxidative damage [83]. The free radicals or ROS do not have specific receptors; therefore, they have a high capacity to damage living cells [81]. Also, they can modify the cell gene expression that could lead

to an increased production of free radicals and ROS [84]. Moreover, these free radicals significantly impact the characteristics of the processed food, such as unfavorable color and flavours [84].

Natural antioxidant peptides can prevent the harmful effects of free radicals and ROS [81]. Natural antioxidants may play role in: 1) scavenging free radicals thus preventing hydrogen atom (H^+) transmission and electron migration, and 2) metal ion chelation (Fe^{2+}/Cu^{2+}), which is responsible for inhibiting hydroxyl radical chain reactions [83]. However, metal ion chelation may work as anti-nutritive agent via binding some important metals that the human body needs such as, zinc and calcium leading to a deficiency in these micro-nutrients [85]. Several chemical structures of the bioactive peptides contribute to their antioxidant's activities, such as their amino acids composition, sequences, molecular weights, charges, and hydrophobicities [86]. The potential antioxidant peptides, that can be derived from caseins and whey proteins of cow, camel, goat, and sheep milk shown in (Table S1; Appendices). The table shows some characteristics of peptides that affect antioxidant activity. Amino acids with ring-structures (i.e. imidazole, indole, pyrrolidine, benzene, and phenol rings such as histidine, proline, tryptophan, phenylalanine, and tyrosine, respectively) of the bioactive peptides provide the best antioxidant activities, because these rings act as significant proton and hydrogen donors [86]. Besides, the hydrophobicity of the amino acids enhances the antioxidant capacity by allowing the antioxidant peptides to enter the target organs through hydrophobic interactions with lipid bilayers of the organ's membrane. At the same time, the peptide's amphiphilic nature supports the radical-scavenging activity by enhancing the solubility of peptides that improve the proton exchange with free radicals [86].

Moreover, the amino acid's charge plays an important role in determining the biopeptide's antioxidant activity. Acidic amino acids with a negative charge (e.g., Glutamic acid and Aspartic acid) have surplus electrons that play roles in stopping oxidative stress [87]. However, the sequences

and steric structures of the amino acid affect the antioxidant capacity of the peptides [87]. The sequences of hydrophilic, hydrophobic, and aromatic amino acids in the peptides determine the overall antioxidant capacity of the peptides [88]. Moreover, the amino acids at the N-terminal of the peptide have been found to be less important than those at the C-terminal [88]. For example, when the histidine is present at the C-terminal, it forms the coordination bond to chelate Fe^{2+} by the imino group of the Histidine' imidazole ring [88]. The amino acid sequences also affect the antioxidant's capacity; such as, when Glu and Tyr are adjacent to each other in the same peptide, the carboxyl group of Glutamic acid encourages the release of the H^+ of the phenolic hydroxyl group of tyrosine [87]. Nevertheless, the low molecular weight may have a significant effect on the antioxidant activities of biopeptides by having more antioxidant peptide's per unit reaction area [89].

Antioxidant bioactive peptides obtained from camel milk and other species milk (i.e., cow, goat, and sheep milk) have been reported in literatures. Most of the identified antioxidant peptides from camel milk are rich in charged amino acids (like glutamic acid, aspartic acid, arginine, and lysine), polar amino acids (mainly, glutamine and asparagine), hydrophobic amino acids (glycine, proline, valine, alanine, leucine, methionine, and isoleucine), and aromatic amino acids (mainly, phenylalanine and tyrosine). When the antioxidant peptides of camel milk are compared with the peptides of other milk with similar functions, similarity in amino acid compositions are observed.

The antioxidant peptides from camel milk whey proteins are more potent than the antioxidant peptides from camel milk casein [90]. This could be due to the distributions of hydrophilic amino acids such as lysine, aspartic acid, glutamic acid, and serine with hydrophobic amino acids in camel milk whey protein's peptides[90]. The residues like GY, GW, and DP are abundant in camel milk whey protein's peptides. These amino acids sequences contribute to the peptide's antioxidant properties by having the extra electrons present in the carboxyl group of the acidic amino acids

like aspartic acid and glutamic acid and by freeing hydrogen atoms from the phenolic hydroxyl group of tyrosine and thus increase the reducing power[91]. Leucine or phenylalanine found at the C-terminal of whey protein's peptides also contribute to the antioxidant activity [91].

2.3. Antihypertensive peptides

In 2019, about 23% of Canadian adults (20 - 79 years old) were diagnosed with hypertension [92]. The blood pressure in the body is mainly regulated by the renin angiotensin aldosterone system. Angiotensin I-converting enzyme (ACE) plays a key role in blood pressure regulation as well as water and electrolytes balance. ACE increases blood pressure by conversion of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) by hydrolyzing the peptide bond between Phe and His [93]. Angiotensin II receptor blockers and angiotensin converting enzyme (ACE) inhibitors are the main types of antihypertensive drugs[94]. The mechanism for most of the antihypertensive bioactive peptides derived from milk are based on the inhibition of ACE [95'96'97'98'99'100'101'102'103'104'105'106'107'108]. A few studies proposed other mechanisms for antihypertensive action such as decreasing aldosterone [109], increasing endothelial vascular function [110], diminishing arterial stiffness [111], and increasing endothelial dilation [112].

Milk derived peptides exhibit antihypertensive property through reducing blood pressure. Several *in vitro* and *in vivo* studies demonstrated that the milk products hydrolysates are a good sources of ACE inhibitory peptides or vasodilators [63,113]. ACE inhibitor peptides that are shown to be effective *in vivo* usually have a short sequence (2-12 amino acids) [104,102,100]. Clinical randomized, single and/or double-blind, placebo-controlled human trials that documented the effect of milk-derived dietary peptides on the blood pressure are summarized in Table 1. These studies used fermented dairy products with a known concentration of VPP and IPP [104,100,107], fermented dairy products supplemented with VPP and IPP [102,106,96], or pure VPP and IPP [95'97]. Studies that

employed fermented milk products used yoghurt-type products fermented with *L. helveticus*[114], a dairy starter culture with a well-characterized proteolytic system. The dose of VPP and IPP ranged from 1.5 and 1.1 mg / person per day to 30 and 23.2 mg/ person per day, respectively (Table 2.1). The selection of study participants included normotensive, moderately hypertensive, and hypertensive subjects and the treatment time ranged from single dose to 24 weeks (Table 2.1).

For example, consuming fermented milk products (Calpis) supplemented with 1.5 mg and 1.1 mg VPP and IPP per day, respectively, showed a reduction in the systolic and diastolic blood pressure by 14.1 ± 3.1 mmHg and 6.6 ± 2.5 mmHg after 8 weeks of treatments with medication in hypertensive subjects, respectively[104]. The blood pressure lowering effect of *Lactobacillus helveticus* LBK-16H fermented milk fortified with 30 mg VPP and 22.5 mg IPP was determined through a randomized, double blinded placebo-controlled study on 94 mildly hypertensive patients for 10 weeks. The systolic blood pressure dropped from 148.4 ± 8.1 mmHg to 132.6 ± 9.9 mmHg, whereas diastolic blood pressure dropped from 93.5 ± 6.2 to 83 ± 8.0 mmHg for the *L. helveticus* fermented cow milk group [102].

Another clinical, randomized, single-blind, placebo-controlled trial was carried out to evaluate the dose-dependent antihypertensive effect of casein hydrolysate tablets supplemented with VPP and IPP on 131 high-normal blood pressure and mild hypertension participants for 6 weeks. Four doses, 0, 1.8, 2.5, and 3.6 mg of VPP and IPP mixture were supplemented through tablets. After 6 weeks of treatment, a dose-dependent decrease in the systolic blood pressure for the active group receiving 1.8, 2.5, and 3.6 mg compared to the baseline and placebo group was observed[100]. Another study to determine the role of diet type (low salt diet) on supporting the VPP and IPP effect, the 24-h ambulatory blood pressure measurements were measured after giving each participant 5.32 mg VPP and 2.76 mg IPP for 8 weeks. This study demonstrated that the systolic

blood pressure was affected by tripeptides and decreased during nighttime sleep after 4 and 8 weeks. Therefore, the low intake of salt could support the antihypertensive activity of VPP and IPP [106].

As shown in Table 2.1, most of the studies were performed to evaluate the antihypertensive effect of casein and whey hydrolysate supplemented with VPP and IPP as beverages or tablets. The impact of dietary intervention with VPP and IPP on the systolic blood pressure differs in magnitude but is largely consistent across the different studies. In contrast, an inconsistent effect on the diastolic blood pressure is observed (Table 2.1). Only a few studies reported outcomes that are related to blood pressure such as endothelial vascular function or arterial stiffness (Table 2.1).

The modest dose-dependent effects were also observed for the systolic blood pressure. The magnitude of the decrease in systolic blood pressure was -14.1 ± 3.1 mg Hg. Most of the studies showed that the antihypertensive effects were greater in normotensive and mildly hypertensive subjects in compared to more severely hypertensive patients. The duration of the dietary intervention does not seem to impact the outcomes related to blood pressure.

None of the studies summarized in Table 2.1 reported any adverse effects of consuming milk hydrolysates and / or purified peptides on human health. This is an optimistic point for using milk bioactive peptides in human studies because safety of nutraceuticals is a necessary feature for regulatory acceptance and successful commercialization. Protein hydrolysates that are obtained by food grade enzymes are generally considered as safe; however, it was indicated that purified peptide fractions or purified peptides may require a safety assessment for approval as novel food [71].

Regarding the antihypertensive activity of bioactive peptides derived from camel milk, camel caseins have similar IPP frequency as bovine caseins but not for VPP (Table 2.1). Camel casein have more proline than bovine milk proteins. Since an N-terminal proline is a key structural determinant of ACE-inhibitory peptides [115] and amide bonds adjacent to prolines are more resistant to proteolysis [116], camel milk may include ACE inhibitory peptides that are not present in bovine milk. ACE inhibitory data for camel milk derived peptides are mostly from *in vitro* studies [44] since the *in vivo* studies are very limited [117]. A RCT study showed no significant differences between fermented camel milk and diluted yogurt from bovine milk on blood pressure and obesity measures on 24 healthy adolescents with mild metabolic syndrome (13.77 ± 1.87 years old) [118].

Table 2.1:Antihypertensive activity of milk-derived bioactive peptides in randomized clinical trials.

Protein/ Peptide (Source)	# In bovine protein	# In camel protein	Treatment period [week] (dose [mg/day])	Effects on blood pressure (▼in SBP/DBP compared to placebo) [ref]
FFVAPFPEVFGK (Bovine)	1(αs1 casein)	0	Single dose (200 mg & 3.51 g alginic acid)	▼(–9.2 ± 3.2/–6.0 ± 2.0)[99]
			4 (3.8 g)	▼(–10.7 ± 1.6 /–6.9 ± 1.2 mm Hg), ▼plasma angiotensin II and aldosterone [109]
Bovine casein hydrolysate with (VPP + IPP)	VPP 1(β-casein) IPP 1(β-casein) 1(κ-casein)	VPP 0 IPP 1(β-casein) 1(κ-casein)	6 (0, 1.8, 2.5, & 3.6 mg VPP+IPP)	▼ (0, –5.8/0, –6.2/0, & –9.3/0 mmHg)[100]
			8 (2.3, 4.6, & 9 mg VPP+IPP)	▼(+0.1/–1.3, –1.5/–1.4, & –2.5/–1.9 mmHg) [101]
			10 (30 mg VPP & 22.5 mg IPP)	▼ (–4. 1± 0.9/ –1.8 ± 0.7)[102]
			4 (18.7 mg VPP & 15.9 mg IPP)	▼ (~ –5.0/0) [103]
			8 (1.5 mg VPP & 1.1 mg IPP)	▼ (–14.1 ± 3.1/–6.6 ± 2.5 mm Hg)[104]
			4 (4.1 mg VPP & 6 mg IPP)	▼ (– 3.4 ± 4.4/–3.1 ± 3.2 mmHg)[105]
			8 (5.32 mg VPP & 2.76 mg IPP)	▼(~ –5.0/–2.0 night sleeping) [106]
			12 (2.26 mg VPP & 1.48 mg IPP)	▼(– 6.1 ± 5.7/–3.8 ± 6.3 mmHg) [107]
			8 (3.4 mg VPP+IPP)	▼(−11.0 ± 11.0/ 0) [108]
			1 (3.42 mg VPP & 3.87 mg IPP)	▲ endothelial vascular function, (▼0/0) [110]
			24; 12 (2.6 mg VPP & 2.4 mg IPP) +12 (26.4 mg VPP & 23.2 mg IPP)	▼ arterial stiffness, especially in metabolic syndrome patients, ▼ (− 4.6/− 2.7 mmHg)[111]

Protein/ Peptide (Source)	# In bovine protein	# In camel protein	Treatment period [week] (dose [mg/day])	Effects on blood pressure (▼in SBP/DBP compared to placebo) [ref]
VPP + IPP (+ plant sterol esters)			10 (4.2 mg VPP+IPP & 2 g plant sterols)	▼ (− 4.1/0 mmHg), ▼total and LDL cholesterol[95]
			Single dose (25 mg VPP+IPP & 2 g plant sterols)	▼ (−2.1/−1.6 mm Hg)[96]
IPP (Bovine)			4 (15 mg IPP)	▼ (−3.8/−2.3 mm Hg)[97]
Whey hydrolysate	—	—	Single dose (20 g Whey hydrolysate)	▲ endothelial dilation[112]
Whey hydrolysate (IW+WL) (Bovine)	IW 1(α-LA) 1(LF) WL 2(α-LA)	IW 1(α-LA) 1(LF) WL 1 (Ig) 1(α-LA)	Single dose (250.5 mg IW & 47.5 mg LW)	▼ plasma ACE activity (0/0)[98]

2.4. Peptides that inhibit starch digestion or improve glucose homeostasis.

Diabetes mellitus (DM), a dominant chronic disease in developed countries, is characterized by an innate insulin secretion deficiency in type 1- diabetes or a defect in the insulin action in type 2-diabetes (T2D). T2D affects ~90% of the diabetes cases, which cause an insufficiency in conveying glucose from the bloodstream into cells, thus increase the glucose level in blood [119]. Persistent hyperglycemia can lead to the development of insulin resistance, and then diabetes mellitus [120]. Delaying carbohydrate digestion is indispensable for the most beneficial treatment of type 2- diabetes. Peptides can delay starch digestion by inhibiting the starch digesting enzymes such as α-amylase and α-glucosidase. Thus, the potential starch digestion inhibitors should have the capacity to bind to the target enzyme's active sites (catalytic sites) via hydrophobic interactions to impede the enzymes arrival to substrates (Figure 2.2) [119,120,68].

The milk derived bioactive peptides exhibit an antihyperglycemic property that diminishes the glucose level in the blood. Numerous *in silico* (Table S2; Appendices), *in vitro,* and *in vivo* studies demonstrated that the peptides from milk product hydrolysates are a good source of antihyperglycemic agents. Clinical randomized, single and/or double-blind, partial and/or complete cross-over, placebo-controlled human trials that documented the effect of milk-derived dietary peptides on the hyperglycemia are summarized in Table 2.2. These studies used milk protein hydrolysates [121], casein hydrolysates [122], whey protein hydrolysates with a known concentration of milk minerals [123], or casein hydrolysates supplemented with leucine[124,122]. The doses of milk protein hydrolysates, whey protein hydrolysates, and casein hydrolysates were 1.4 and 2.8 g [121], 50 g [123], and 0.3 g / kg body weight [124] and 17.6 g [122] / person per day, respectively

(Table 2.2). The study participants included were normal healthy, prediabetic, and type 2- diabetes subjects and the treatment time ranged from single dose to 6 weeks (Table 2.2).

Co-ingestion of casein hydrolysate beverage (0.3g / 4 mL water/kg body weight) enriched with leucine (L) (0.1g / 4 mL water/ kg body weight) after each main standardized meal reduced the prevalence of hyperglycemia significantly with a substantial reduction in the average of 24-h blood glucose concentrations in the T2D patients compared to placebo group. The 24-h blood glucose concentrations of the test group and placebo group were 9.6 ± 0.6 and 10.8 ± 0.5 mmol/l, respectively ($P < 0.05$) [124].

Another study assessed the impact of casein hydrolysates as a single meal replacement on postprandial glucose concentration, serum glucagon, and insulin for T2D patients. Each patient received four types of treatment, specifically, placebo (control), casein hydrolysates (17.61 g), casein hydrolysates plus leucine (17.61 g and 5 g respectively), and unhydrolyzed casein (15 g). The results showed that both casein hydrolysates and casein hydrolysates with leucine supplementation had a similar postprandial glucose concentration reduction of 4.7% compared to 1.7% and 1.6% for unhydrolyzed casein and placebo, respectively. Glucagon concentrations increased by 14% for all treatments compared to the placebo. The casein hydrolysates plus leucine treatment achieved the highest increase in insulin [122].

A monocentric, three-way-cross-over, randomized, placebo-controlled, and double-blind study was performed on prediabetic subjects to determine the α-glucosidase inhibitory activity of whey protein hydrolysates rich in arginine -proline (AP) dipeptide. The tested products were provided in capsules, and each capsule had 350 mg whey protein hydrolysate (include ~ 0.96 mg of AP dipeptide) [121]. In a single dose experiment, after 10 h overnight fasting, participants received a single dose of placebo, a low dose of whey protein hydrolysate peptides (1400 mg), or a high dose

of whey protein hydrolysate peptides (2800 mg) 15 minutes before having a challenge meal rich in carbohydrates (standardized to 75 g of CHO). After a one-week washout period, an open-label single arm design was applied in the experiment of 6 week, and participants received a low dose of whey protein hydrolysate peptides (1400 mg) daily 15 minutes before having a challenge meal rich in carbohydrates. The incremental areas under the concentration–time curves of glucose were significantly reduced by the low dose of whey protein hydrolysate peptides (1400 mg) compared to placebo. However, the longer period of treatment did not have any additional postprandial glycemic effect [121].

Another branched study showed that whey protein hydrolysates plus milk minerals beverage elevated GLP approximately ninefold compared to other beverages. Whey protein hydrolysates plus milk minerals beverage produced ~25% of GLP more than whey protein hydrolysates beverage. No significant differences between milk minerals beverage compared to the placebo was observed [123].

Table 2.2 shows the studies that were carried out to evaluate the beneficial antihyperglycemic effect of casein hydrolysates supplemented with leucine [122,124], whey protein hydrolysates with a known concentration of AP [121], and calcium-enriched milk minerals supplemented with whey protein hydrolysates as beverages or tablets [123]. Only one study assessed the effect of casein hydrolysates compared to intact casein protein and placebo [122]. These studies determined the antihyperglycemic effect based on different parameters like, postprandial glucose concentration, serum insulin, GLP, GIP, and PYY. The degree of effects of casein and whey protein hydrolysates on the postprandial hyperglycemia differs but is largely consistent across the different studies. However, the effect of whey protein hydrolysates is much higher than the effect of casein protein hydrolysates and is due to the high content of leucine and proline amino acids in whey protein hydrolysates.

Table 2.2 demonstrates that co-ingestion of whey protein hydrolysates clearly stimulates plasma GLP, increases the insulin concentration, and decreases the blood glucose concentration. Addition of calcium-enriched milk to whey protein hydrolysates remarkably support high plasma GLP concentrations. Enriched casein protein hydrolysates with leucine increase plasma insulin level. None of the studies that are summarized in Table 2.2 reported any significant adverse effects of milk protein hydrolysates consumption on human health, except only a few subjects claimed gastrointestinal-related abdominal cramps with or without diarrhea. There was no linear dose-response relationship and there were no minimum effective doses of the hydrolysates. However, there was a clear impact of hydrophobic amino acids leucine and proline availability on antihyperglycemic activity of hydrolysates observed in this study.

The inhibitory effect on the activity mainly comes from the amino acid compositions (hydrophobic content) of the peptides itself. However, the inhibitory effects of dipeptidyl peptidase-4 (DPP4), α-amylase, and α-glucosidase enzymes depend on competitive direct interaction to the active binding sites of hydrophobic enzymes and catalytic triad against the substrate (Figure 2.2) [68,120]. Clinical studies listed in table 2.2 emphasized that the proposed mechanisms for antihyperglycemic activity are reduced starch digestion through anti-α-glucosidase activity by milk protein hydrolysates rich in leucine, and/or amino acid-induced gut hormones secretion like increased insulin due to the insulinotropic effect of the peptides and stimulation of the plasma GLP because of peptides composition and calcium enrichment.

Because the sequence information of the peptides with antihyperglycemic activity in bovine milk is limited, it is very difficult to predict which peptides in camel milk protein hydrolysates are responsible for inhibiting the digestion of starch. However, camel milk proteins have more hydrophobic amino acids such as leucine and proline in their sequence compared to bovine milk

proteins. Several peptides obtained by hydrolysis of camel milk proteins were shown to inhibit amylase and glucosyl hydrolases *in vitro*, however, *in vivo* studies with rodent models for diabetes used milk proteins rather than protein hydrolysates or defined peptides (for review, see previously published reviews [125,126]). Likewise, the RCTs that investigated camel milk efficacy in diabetic patients used an experimental design that does not allow conclusions as to whether the observed effects are attributable to bioactive peptides that are released during digestion or not. Moreover, no active peptides were determined and all the clinical studies that claimed the antihyperglycemic activity of camel milk used different volumes of whole camel milk with various treatment times as doses [125,126].

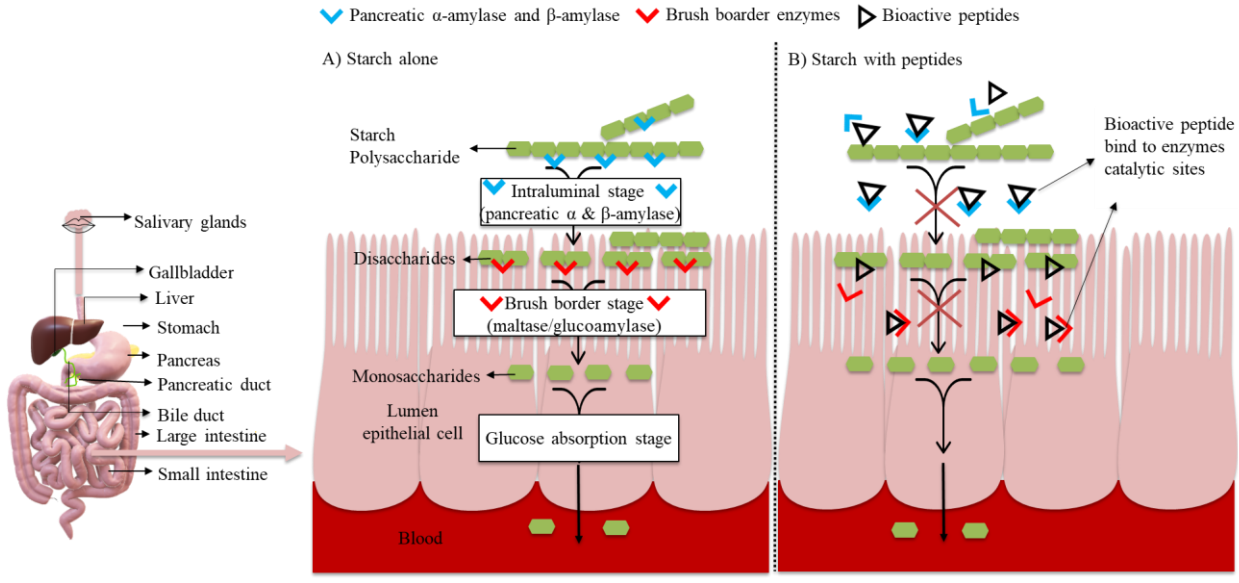


Figure 2.2:Starch digestion in gastrointestinal tract and potential mechanism of milk derived bioactive peptides for starch digestion delaying.

Table 2.2: Antihyperglycemic activity of bioactive peptides in randomized clinical trials

Protein/ Peptide (Source)	# In bovine protein	# In camel protein	Treatment period (dose)	Effects [ref]
Casein hydrolysate (Bovine)	—	—	Single dose (17.61 g ca. hy.)	▼ postprandial glucose values and ▲postprandial insulin response[122]
Casein hydrolysate + Leucine (Bovine)	—	—	3 doses/ day ((0.3 g ca. hy. & 0.1 g L)/ kg body weight)	▼ hyperglycemia in T2D patients over 24 h[124]
			Single dose (17.61 g ca. hy. & 5 g L)	▼ postprandial glucose values and ▲postprandial insulin response[122]
Milk hydrolysate	—	—	6 weeks (1.4 wp. hy.)	▼ plasma glucose after high carbohydrate meal and HbA1c[121]
			Single dose (1.4 & 2.8 g wp. hy.)	▼ plasma glucose after high carbohydrate meal[121]
Whey hydrolysate +milk minerals	—	—	Single dose (50 g wp. hy. & 1000mg Ca.)	▲ plasma GLP[123]

2.5. Antiadhesion activity of glycomacropeptide (GMP).

Caseinomacropeptide (CMP) is the third most abundant protein in cheese whey, constituting about 15-20% of the total whey proteins. CMP represents the C-terminal of κ-casein obtained by the hydrolysis of milk protein by rennet [127]. Hydrolysis of the bovine milk κ-casein at Phe^{105}-Met^{106} linkage releases a polar polypeptide and a non-polar polypeptide. The former is the para-κ-casein, which consists of 105 amino acids and stays in the cheese curd. The latter is the caseinomacropeptide, which consists of 64 amino acids (Met^{106} - Val^{169} residue) and remains in the whey[128]. GMP is produced commercially from bovine whey [129,130]. It was reported that sialic acid linked to κ-casein of bovine milk supported growth of *Bifidobacterium* bacteria [131,132]. GMP is a very good source of sialic acid which constitutes 7% - 9% of the total GMP [133].

CMP has a unique chemical structure and functional properties. CMP is rich in amino acids such as proline, serine, glutamine, and threonine. However, CMP does not have any aromatic amino acids (tyrosine, phenylalanine, and tryptophan) or cysteine [129,134]. CMP is also rich in branched-chain amino acids (leucine, isoleucine, valine) (Figure 2.3). The presence of two aspartic acid and 7-8 glutamic acids makes glycomacropeptide an acidic peptide [134,130]. The bovine milk non-glycosylated CMP have two main variants of κ-casein, A and B with molecular weights 6.75 kDa and 6.78 kDa, respectively (Figure 2.3). The average molecular weight of glycosylated CMP is 7.500 kDa [129]. CMP characteristics are affected by glycosylation and phosphorylation modifications. It has been reported that the glycosylation and phosphorylation of GMP occurs at serine and/or threonine residues at multiple positions (Figure 2.3) [134,135,136].

GMP has multiple biological functions that are conferred by the oligosaccharide's chains attached to each of the GMP portions. In addition to the composition of the oligosaccharides, frequency

and spacing of glycan on the peptide backbone (i.e., increase glycosylation sites) were also shown to affect the biological activity [133,137,138,139].

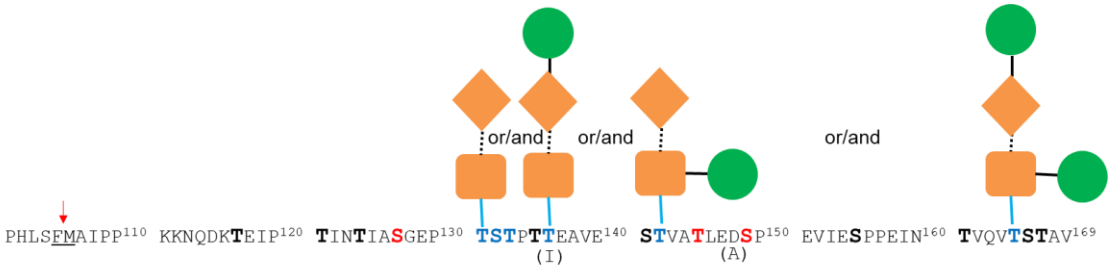


Figure 2.3:Amino acid sequence with glycan structure of glycomacropeptide (GMP) derived from bovine milk (variant A and B). The differences between variant A and B are two amino acid residues at 136 and 148 (variant B shown between brackets). The bold amino acid abbreviations (letters) indicate potential glycosylation sites. Bold, blue-colored letters correspond to reported glycosylation sites, while red-colored letters correspond to reported phosphorylation sites. The red arrow and underline indicate the chymosin's cleave sites between P^{105}-M^{106}. Orange squares, N-acetylgalactosamine; orange diamonds, galactose; green circle, N-acetylneuraminic acid (sialic acid); black dotted lines, β-(1→3) glycosidic linkage; black solid lines; α-(2→3) or α-(2→6) glycosidic linkages; blue lines, link to threonine or serine residues on the peptide backbone. The peptide sequence of the GMP is from UniProt (https://www.uniprot.org/uniprot/) accession numbers P02668, while the numbering of residues is based on the sequence of the κ-casein without precursor [140,141,142,136].

GMP-derived from bovine milk contains galactose (Gal), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid (NeuNAc). These constituent monosaccharides give rise to the oligosaccharide structures that are linked by *O*-glycosylation to the peptide backbone: monosaccharide: (GalNAc), disaccharide: Gal-β-(1→3)-GalNAc), trisaccharides: NeuAc α2 –

3Gal β 1 – 3 GalNAc) and (Gal β 1 – 3 (NeuAc α2 – 6 GalNAc), and tetrasaccharide: (NeuAc α2-3 Gal β 1 - 3 (NeuAc α2 – 6 GalNAc) (Figure 3)[133,143,144]. Glycosylation with oligosaccharides that additionally include fucose and N-acetylglucosamine (GlcNAc) were also reported in GMP from bovine colostrum [133].

In addition to providing a dietary source of sialic acid, biological activities of GMP include substrate for intestinal bacteria including bifidobacteria and the inhibition of pathogen adhesion [145].

The inhibition of pathogen adhesion is well supported by *in vitro* and *in vivo* studies (Figure 2.4). Most of enteric pathogens including *Salmonella*, enterotoxigenic *Escherichia coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), *Shigella flexneri, Helicobacter pylori*, enterotoxins LT-I and LT-II derived from *E. coli.,* and cholera toxin adhere by glycan recognition to infect or invade the host cells [146,147,74,148,149]. For instance, ETEC are a major cause of childhood diarrhea in developing countries and cause traveler's diarrhea. ETEC K88 cause watery diarrhea in newborn and post-weaning piglets and calves [150,151,152]. ETEC adhere to the small intestinal epithelial cells and to the mucosal tissue through glycoprotein receptors of the host cells using specific fimbriae and colonizes in the microvilli that lead to electrolytes imbalance and water loss (Figure 2.4) [153,154].

Glycan receptors are glycolipids or glycoproteins on the surface of the host tissues that mediate adhesion of pathogens and toxins including the adherence of ETEC fimbriae to the epithelial cells. Glycan receptor analogues bind to bacterial lectins and thus inhibit the initial stages of infection and bacterial colonization (Figure 2.4) [155,156]. For example, K88 fimbriae mediate the binding of *E. coli* ECL13795 to glycan receptors [152]. Porcine aminopeptidase N is a receptor for K88 fimbriae; in addition, surface glycan oligosaccharides composed of GalNAc, GlcNAc, galactosamine, and N-acetylmannosamine were proposed as receptors for ETEC K88 adhesion [152,157].

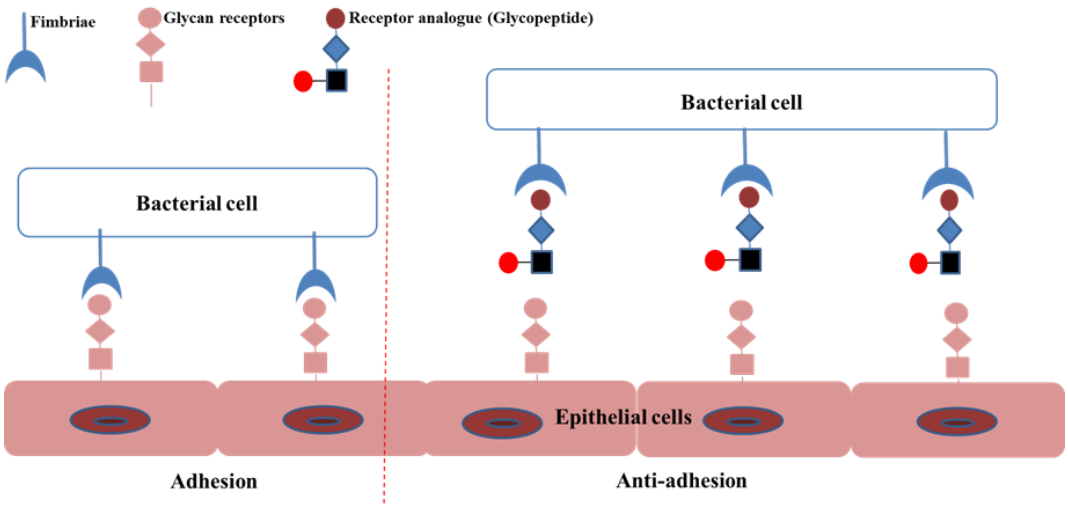


Figure 2.4: Adhesion of bacteria to the epithelial cells and antiadhesion activity for glycopeptides.

Anti-adhesion biomolecules that act as glycan receptor analogues could be a promising alternative to antibiotics. Anti-adhesion agents are not antibacterial agents and thus do not lead to the development of antimicrobial resistance [158]. One of the main biological properties of GMP is anti-adhesion activity against enteric pathogens. *In vitro* studies demonstrated the potential anti-adhesion activity of GMP against enteric pathogens to the intestinal mucosa including enterohemorrhagic *E. coli* (EHEC) O157, ETEC K88, *Salmonella enteritidis*, *Salmonella typhimurium*, *Helicobacter pylori*, and *Shigella flexneri* [146,147,74,148,149]. On other hand, several *in vivo* studies confirmed the anti-adhesion activity of GMP against enteric pathogens in farm animals [75,76,159,148] .

For example, the anti-adhesion activity of glycoprotein glycans against the ETEC K99 in the calf was confirmed *in vivo*. Directly after birth, colostrum was administered to calves, at age of 2-8 hours, and then 10^7-10^{10} CFU of ETEC K99 was administered orally to the calves. When the first sign of diarrhea appeared, 250 mg of oligosaccharides was ingested orally every day for three days.

The adhesion of ETEC K99 to the small intestine was significantly reduced in the calves treated with oligosaccharides compared to control [76]. Moreover, the anti-adhesion activity of GMP against the ETEC K88 was confirmed by inclusion of GMP in the diet of weaning piglets challenged with ETEC K88 (1 and/or 2%; 10 [159] and/or 20 [75] g/ Kg dry matter of diet). A significant reduction in ETEC adhesion to the intestine epithelial cells and reduced overgrowth of ETEC in digestive tract was observed in the challenged treated group [159,75]. A dose-dependent protective effect of GMP to neutralize the toxicity of *Cholera* toxin and enterotoxins LT-I and LT-II derived from *E. coli* was also confirmed in mice. Oral administration of 0.2, 0.5, and 1.0 mg/day/mouse for 7 days before challenge of mice with toxins led to a significantly decreased rate of induced diarrhea[148].

The differences in the topological spacing of glycans and glycan content are recognized as important factors affecting the anti-adhesion activity of glycopeptides [133,137,138,139]. Camel milk is not as studied as bovine milk and the chemical compositions and the biological activities of camel CMP and GMP are not reported. To our best knowledge. there is no information about anti-adhesion activity of CMP and GMP from camel milk available until now.

2.6. Conclusions.

In summary, numerous *in vitro* and *in vivo* studies confirmed the efficacy of bovine milk-derived bioactive peptides against blood hypertension, postprandial hyperglycemia, and anti-adhesion activity against enteric pathogens. However, the clinical data on these activities of milk peptides are very limited. Moreover, the research on camel milk is also very limited in compared to that involving bovine milk. Most of the research has focused on raw milk only and whey protein from camel milk has been overlooked. There are no active structures of peptide, and only a few clinical studies have addressed the antihyperglycemic activity of camel milk. Whey is a by-product of

dairy industry and has been considered as an inexpensive source of high-nutritional quality protein and bioactive peptides for the food and health industries. Therefore, to explore the potential bioactive peptides from camel milk designed *in vivo* studies on camel milk-derived protein hydrolysates are required. Further, additional RCT trials are required to evaluate the full potentials of bioactive peptides derived from milk, and to determine the bioavailability of ingested bioactive peptides.

Chapter 3. **Antioxidant properties of *in vitro* digests of flavourzyme-treated camel milk whey protein hydrolysate**

-A draft of this chapter is in preparation as a manuscript for publication.

3.1. Introduction

Milk whey is a by-product of the cheese production process and is considered waste in developing countries. Due to the consumption of cheese products increasing worldwide, the cheese manufacturers are having difficulties accommodating the high volume of whey [79,160]. Whey contains approximately 20% of the original milk protein and represents a significant source of protein. Therefore, whey could be an inexpensive source of high-nutritional quality protein for the food and health industries [79,48].

Whey contains a rich mixture of soluble proteins with diverse physical, chemical, and functional properties. Preliminary investigation of many whey proteins revealed potential health benefits[161].Thus, more scientific research and evaluation on whey proteins is necessary to understand how best to use it as a dietary supplement or food additive [48].

Camel milk is broadly consumed in the Asia and Africa countries. Many processed milk products such as cheese, butter, chocolate, yogurt, ice cream, and fermented milk from camel are available in the marketplace [162]. Thus, a substantial amount of whey is available from camel milk production [45]. Camel milk contains about 25% of whey protein and α-lactalbumin is the main constituent of whey protein; notably, is the absence of β-lactoglobulin. Whey also contains lysozyme, lactoferrin, serum albumin, lactoperoxidase, peptidoglycan recognition proteins, and immunoglobulins [162]. Camel milk whey proteins contain most of the essential amino acids (i.e., Phe, Val, Thr, Try, Met, Leu, Ile, Lys, and His), many of which are in a high concentration of (Phe, Val, Leu, and Lys). On the other hand, the non-essential amino acids of camel milk whey protein are present in low

concentrations except Glu and Pro. These inherent characteristics of camel milk whey protein make it a promising candidate for the production of bioactive peptides that can be a major part of functional food [163].

The proportion of casein and whey proteins in camel milk is different than that in milk from other species [163]. While the ratio of casein and whey proteins in bovine, ovine, and caprine milk is ~80%:20%, this ratio in camel milk is ~75%:25% [164,165]. In the last century, camel milk attracted attention due to the biological and therapeutic properties of camel milk components [43]. Camel milk contains many compounds with antioxidant, antihypertensive, and antihyperglycemic properties which may be able to improve and promote human health when included in a balanced diet [166].

Most of the antioxidant peptides from milk studied are limited to cow milk [44]. Only a handful of studies have investigated the antioxidant peptides from the milk of other mammals like sheep, goats, horses, and donkeys. Very rare research on camel milk studied the antioxidant activities, and most has focused only on raw milk, casein, and fat[44]. Only recently whey protein from camel milk has attracted attention and studies on it have been initiated [42].

The oxidation reaction is a natural metabolic process in living organisms, but uncontrolled oxidation as indicated by an imbalance between oxidation and reduction often produces free radicals or reactive oxygen species (ROS) [167]. Natural antioxidant peptides can prevent the harmful effect of free radicals and ROS through scavenging the free radical or metal ion chelation [167]. Bioactive peptides derived from natural sources through enzymatic hydrolysis and fermentation can be used to prevent uncontrolled oxidation [3].

Due to the lack of information on the antioxidant properties and processing procedures of the camel milk whey proteins (CMWP), studies are needed to investigate gastrointestinal enzymatic digestion and to develop hydrolysis techniques that produce bioactive peptides from whey protein with known antioxidant activity. The aim of this study, therefore, was to assess the properties of

the peptides derived from hydrolysis of camel milk whey protein with different enzymes and a mixture of enzymes (Neutrase, Flavourzyme, Alcalase, and the mixture of Neutrase and flavourzyme) and the digestive enzymes (pepsin and trypsin) and to assess the effect of this hydrolysis process on the antioxidant activities of the produced peptides.

3.2. Materials and methods

Materials

Powdered camel milk (Chinese Bactrian camel) was provided freeze dried by the Food Science Department at the Inner Mongolia University, Hohhot, China. Hexane (EC:203-777-6), neutrase (≥0.8 U/g; EC: 232-752-2), alcalase (EC: 3.4.21.62), pepsin (800-2500 U/mg protein; EC: 3.4.23.1), trypsin (EC: ≥250 U/mg; 3.4.21.4), flavourzyme (500 U/g; EC: 232-752-2),1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, the fluorescent dye 8-anilino-1 naphthalene sulfonic acid (ANS), Trinitrobrnzenesulfonic acid (TNBS), 3-)2-pyridyl)-5,6 bis(4-phenyl-sulphonic acid)-1,2,4- triazine (Ferrozine) were acquired from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) All reagents used were reagent grade.

Camel milk whey protein isolation

About 500g of camel milk powder were defatted by dispersing powder into hexane at a powder to hexane proportion of 1:10 (w/v) and stirring vigorously (1000 rpm, 23˚C) in a fume hood for 24 h. The suspension was then centrifuged at 7,500g for 30 min at 4˚C (Beckman Coulter Avanti J-E Centrifuge System, CA, USA). The supernatant was stirred vigorously (1000 rpm, 23˚C) in a fume hood for a further 2 h and then centrifuged again under the same conditions. The supernatant realized after the second centrifugation was stirred and centrifuged using the same conditions to completely extract fat and some other compounds from the camel milk. The precipitate was collected, spread into a dish, and placed in a fume hood to allow the hexane to evaporate at ambient

temperature (23˚C) for 24 h. A dry powder resulted, and the obtained defatted camel milk powder was lyophilized (E-9320, LABCONCO) for 4-5 days. After lyophilization, the defatted dry powders were stored in plastic bottles at -21˚C until the separation process.

Depending on protein contents as determined using a nitrogen analyzer (FP-428, LECO Corporation, St, Joseph, MI, USA), skim camel milk solution was reconstituted by dissolving defatted camel milk powder into water at a powder to water ratio of 1:11 (w/v) with vigorous stirring (1000 rpm, 23˚C) for 2h. The milk solution was then heated to 37˚C and the pH value was adjusted to 4.6 using 6 M HCL. The solution was kept at 37˚C for 30 min, and the caseins were precipitated and then isolated from the transparent supernatant containing whey protein by centrifugation at 10,000g for 60 min at 4˚C (Beckman Coulter Avanti J-E Centrifuge System, CA, USA). The latter step was repeated 3 times. Once the whey protein was collected, it was then dialyzed in distilled water using a membrane with a molecular weight cut off 3.5-5 kDa with stirring (125 rpm, 4˚C) for 7 days to increase the protein content, with distilled water changed every 4 h. Whey protein was purified using an ultrafiltration unit (Centromere PE, Pall Life Sciences, Mississauga, ON, Canada), with a membrane molecular weight cut off 10 kDa.

Nitrogen content of the concentrated whey protein was determined using a nitrogen analyzer (FP-428, LECO Corporation, St, Joseph, MI, USA). Then, the whey protein concentrate was lyophilized and stored at -20˚C until further analysis.

Camel milk whey protein enzymatic hydrolysis

A 1% whey protein in 0.02 M phosphate buffer solution (PBS) was prepared by solubilizing the lyophilized whey protein at 23°C for 0.5 h with stirring, with the pH of each solution suitable for the enzyme used (Table 3.1). Each whey protein solution was allowed to equilibrate at the hydrolysis temperature appropriate for the enzyme to be used (Table 3.1) for 30 min. Neutrase,

flavourzyme, alcalase, and a neutrase (0.5%)- flavourzyme (0.5%) mixture were then incorporated into the 1% whey protein solution at 1% (w/w) and allowed to hydrolyze the whey protein under reaction environments that were ideal for each enzyme (Table 3.1). Hydrolysis was performed for 30, 60, 90, 120, 180, and 240 min with vigorously stirring. The temperature and pH of each hydrolysis mixture was monitored and optimized every 20 min during hydrolysis (Table 3.1).

Table 3.1: The optimum conditions for enzyme used in whey protein hydrolysis

Enzyme	Optimum Temperature	Optimum pH
Neutrase	50 °C	7
Flavourzyme	50 °C	6.6
Alcalase	55 °C	8
Neutrase-Flavourzyme	50 °C	7

The hydrolysis reaction was stopped by holding each whey protein solution at 95 ºC for 12 min and then each hydrolysate was centrifuged at (5000 ×g 10 min at 23 ºC) (Beckman Coulter Avanti J-E Centrifuge System, CA, USA) to collect the supernatant. The supernatants were lyophilized and stored in a plastic bottle at −20 °C for further analysis.

Degree of hydrolysis (DH)

The degree of hydrolysis was determined using the methods of Silvestre 1997. Hydrolysate was incubated with 20% trichloroacetic acid (TCA) at a ratio of 1:1 for 1 hour at 4°C, and then centrifuged (2000 x g, 10 minutes, 4°C). The supernatant absorbance was then read spectrophotometrically (model V-530, Jasco, CA, USA) at 280 nm to determine the degree of

hydrolysis through the detection of soluble free amino acids and small peptides concentration in solution[168] . The degree of hydrolysis (DH%) was calculated as:

DH%= {(mg soluble protein after hydrolysis/mL – mg soluble protein before hydrolysis/mL)/mg of soluble protein in starting solution} x 100%; with DH% and expressed as percent.

Antioxidant properties

DPPH scavenging activity

The procedure of Tang et al. 2009 was used to determine the DPPH scavenging activity. Briefly, aliquots of hydrolysates with protein concentration (1g/L) were mixed with 0.1 mM DPPH at a ratio 1:1 (v/v), the combinations were agitated for 30 min at ambient conditions and the mixtures were kept in darkness to limit photo-oxidation. Synthetic antioxidant and natural (ascorbic acid) were used as controls. DPPH scavenging activity was detected via scaling the absorbance at 517 nm (UV-visible spectrophotometer, model V-530, Jasco, CA, USA). DPPH free radical scavenging activity was calculated using the equation:

%DPPH free radical scavenging activity = 1- (As/Ac) ×100 Where, As and Ac represent the absorbances of the sample and the control (deionized water instead of hydrolysates), respectively.

Superoxide radical (O^{2-}) scavenging activity

The method of Tang and other[169] was used to determine the superoxide radical scavenging ability by estimating the suppression of pyrogallol autoxidation. This method considers the reduction ability of experimental substances dependent on O^{2-} reaction resulting in the production of chromophoric substances. Eighty (80) µL of hydrolysate at 0.5 mg/ml protein concentration were mixed with 80 µL of 0.5 M Tris-HCL buffer (pH 8.3) in a 96-well micro plate and 40 µL of 1.5 mM pyrogallol in 10 mM HCL were added with mixing. The polymerization of pyrogallol stimulated by O^{2-} (ΔAs/min) was determined according to the elevation in absorbance at 320 nm

at 23 ºC for 5 min against a control of 0.01 and 0.1 g/L BHT and a blank of 50 mM Tris-HCL buffer. The O^{2-} scavenging activity was calculated using the following equation:

O^{2-} Scavenging activity = $[\Delta A_c/min) - (\Delta A_s/min)] / (\Delta A_c/min) \times 100$

Where, As and Ac represent the absorbances of the sample and the control, respectively

Reducing power

The reducing power was determined according to Oyaizu [170] method. One (1) mL of hydrolysate with protein concentration (1 g/L) was added to 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% reducing power of the hydrolysate's potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min, and then 2.5mL of 10% TCA were added to stop the reaction. After centrifugation at 5000g, at 23 ºC for 10 min, the supernatant was retained, and 2.5 mL of the supernatant was diluted with 2.5 mL deionized water and 0.5 mL of 1 g/L $FeCl_3$ in a test tube. After 10 min, the absorbance of the resulting solution was measured at 700 nm. Samples were measured against a blank where distilled water was substituted for sample. The increment in absorbance of reaction mixture indicated the increment of hydrolysates' reducing power activity.

The ferrous (Fe^{2+}) Chelating activity

The ferrous (Fe^{2+}) chelating activity of hydrolysates was determined as described by Kong and Xiong[171]. Briefly, 0.5 ml hydrolysate with protein concentration (1g/L) was mixed with 1.0 ml of $FeCl_2$ ($0.2 \times 10^{4-}$M, 20µM) and 1 ml of ferrozine ($0.5\times 10^{3-}$M, 0.5 mM), agitated and then stored at ambient conditions for 15 min. The absorbance was then measured at 562 nm (A_s). EDTA (0.1 mg/mL), a strong metal chelator, was used as a positive control. The chelating ability of the hydrolysates was calculated according to the following equation:

Chelating ability (%) = $(A_c - A_s)/A_c \times 100\%$

Where A_c and A_s represent the absorbance of the sample and the control (deionized water instead of hydrolysate), respectively.

In vitro gastric and small intestine digestions

The digestion process was modelled as described by Minekus et al. (2014)[172]. Briefly, the solution of the hydrolysate was achieved the highest antioxidant activity (flavourzyme at 1.5 h) with 1% protein concentration was prepared. Gastric *in vitro* digestion process was carried out with stirring (375 rpm) in 250 mL Schott bottles immerged in a jacketed beaker filled with water heated to 37 ºC. The simulated digesta was adjusted to pH 2 using 1M HCL and pepsin was then added at 2000 U/ml of the final digestion mixture. Hydrolysis was performed for 0.5, 1 and 2 h, and then the digesta pH was adjusted to 8 using 1M NaOH and trypsin was added at 100U/ml of the final digestion mixture. Hydrolysis continued for 3, 4 and 6h with vigorous stirring. Hydrolysis was stopped by heating the digesta at 95ºC for 12 min. The hydrolysates were lyophilized and kept in a plastic bottle at -20ºC for further analysis.

Digesta antioxidant capacity

The antioxidant capacities of the digesta were tested as described in Section (3.2.5). The digest with the highest antioxidant activities were lyophilized and kept at -20ºC for further analysis.

Digesta molecular weight

The average molecular weight (Mw) of FH during digestion was determined by size-exclusion high-performance liquid chromatography (SE-HPLC, Agilent series 1100, Palo Alto, Ca, USA) equipped with a TSK G3000 SW column (5 µm, 7.8 mm ID ×30 cm; Tosoh Bioscience, LLC, Japan) at 22ºC. The mobile phase was 0.1 ml/min. Absorbance signal was detected by UV detector at 280 nm. The protein percentage was 1 mg/ml and the samples were filtered using a 0.22 µm filter before injection into the column [173].

Statistical Analysis

The antioxidant activity and structural characterization for all hydrolysates were performed in triplicate biological repeats with three technical repeats, and the mean of three replicates ± standard deviation (SD) was presented in tables and figures. Statistical significance of the differences was estimated by LSD: least significant difference ($p < 0.05$).

3.3. Results

Degree of hydrolysis (DH%):

The degree of hydrolysis is expressed as a percentage (DH%) of the produced hydrolysates with the linear hydrolysis progressed rate in the first 0.5h of hydrolysis for all enzymes and the overall hydrolysis rate is shown in Figure 3.1. Flavourzyme hydrolysates achieved the highest degree of hydrolysis among all enzymatic hydrolysates after 1h of hydrolysis time, and the highest value was 7.6% at 4h.

Impact of enzymatic hydrolysis on antioxidant properties of camel milk whey protein

The antioxidant activities of the camel milk whey protein (CMWP) hydrolysates were determined by four independent tests: 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, superoxide ($O^{\cdot 2-}$) free radical scavenging, ferrous ion (Fe^{2+}) chelating activity, and reducing power activity (Figure 3.2). The lowest effective concentrations of bioactive peptides were selected to use in each test. Figure 3.2A shows the free radicals scavenging activity of DPPH.

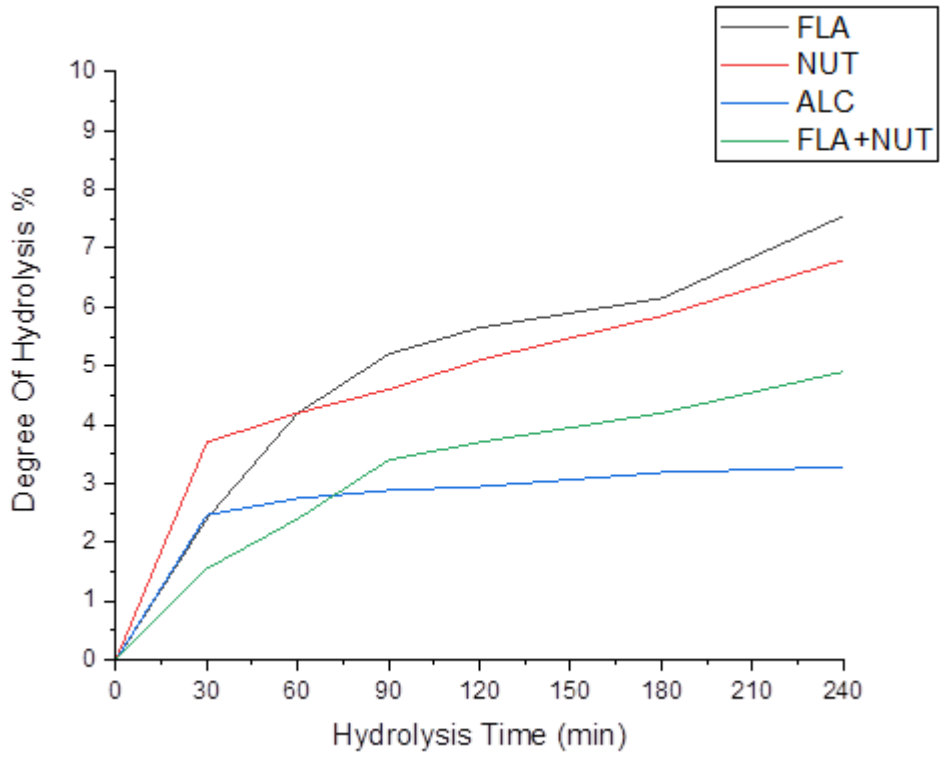


Figure 3.1. Degree of hydrolysis determined using TCA acid (20%): whey proteins hydrolysis by different proteases at 30, 60, 90, 120, 180, and 240 min.

Figure 3.2A shows that DPPH free radical scavenging activity for all CMWP proteases hydrolysates were differentially affected by hydrolysis times. Flavourzyme hydrolysates (FH) displayed a sharp increase in scavenging capacity at 1.5h of hydrolysis time and had the best DPPH free radical scavenging activity (71.47%) compared to other hydrolysates at different incubation times.

The superoxide free radical scavenging activity of CMWP hydrolysates at different hydrolysis times is presented in Figure 3.2B. It shows that there was no clear relationship between hydrolysis time and superoxide radical activity for all hydrolysates regardless of the proteases used. However, FH exhibited the maximum superoxide scavenging activity at 1.5 h, with a gradual decrease in

superoxide scavenging activity after that. Alcalase hydrolysates (AH) exhibited the maximum level of superoxide scavenging activity at 1 h, While Neutrase hydrolysate (NH) and Neutrase-Flavourzyme hydrolysate (N+FH) hydrolysates exhibited the maximum scavenging activity at 3 h. Overall, FH showed the maximum superoxide scavenging activity at 1.5 h (45%) compared to the other enzymes.

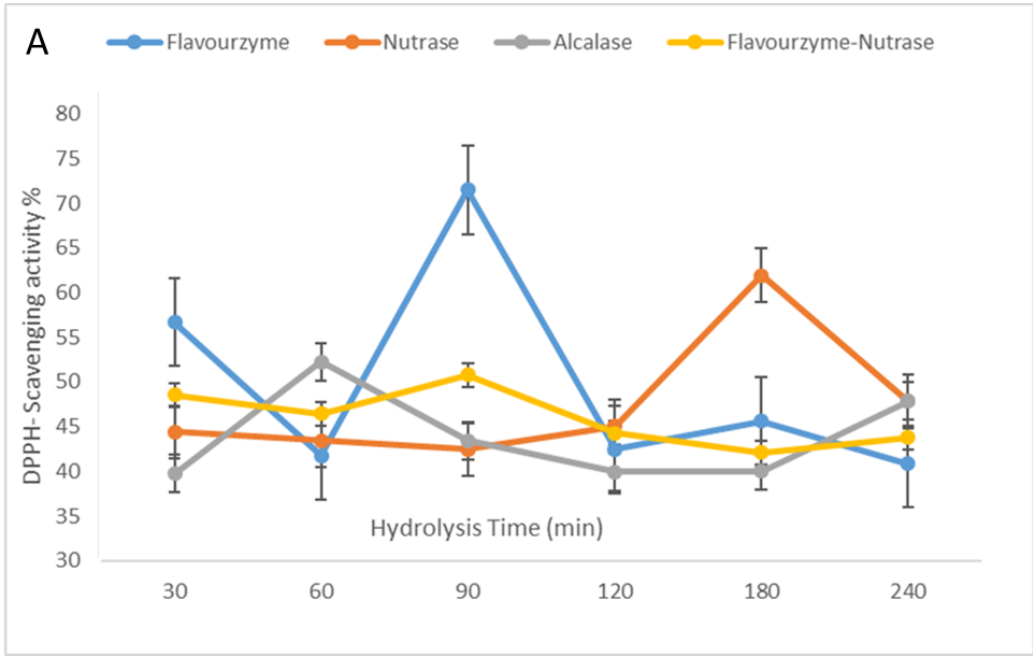
The pattern of ferrous ion (Fe^{2+}) chelating activity for CMWP hydrolysates is illustrated in Figure 3.2C. The figure shows that all hydrolysates possessed high Fe^{2+} chelating capacities, ranging from 65.61% to 71.94%. At the beginning of hydrolysis, there were slight increases in the Fe^{2+} chelating activity of the AH and FH hydrolysates, with AH achieving the maximum value at 1 h (71.94%), whereas FH reached its top value at 1.5h (71.54%). The other proteases hydrolysates NH and N+FH exhibited their maximum Fe^{2+} chelating activity at 3 h of hydrolysis. Figure 3.2D illustrates the reducing power of CMWP hydrolysates as measured by the redox-linked colorimetric reaction[173]. It can be seen from the figure that the reducing power increased in the first 1.5 h for the FH protease hydrolysate, and then decreased sharply. NH and AH reached their top reducing power at 3h, and the (N+FH) achieved the highest reducing power at 0.5 h. The FH hydrolysates at 1.5 h achieved the maximum reducing power among all enzymatic hydrolysates (0.045).

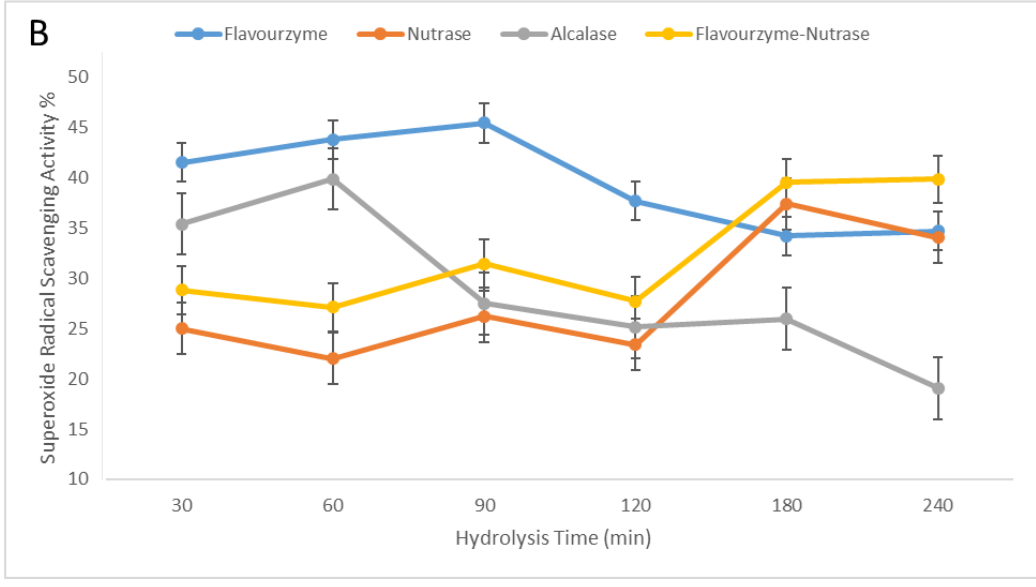
Considering the previous results for all antioxidant activity assays, it has been noticed that the type of enzymes is the main determinant in defining the antioxidant capacities of camel milk whey protein hydrolysates. Since the antioxidant activity of FH at 1.5 h hydrolysis is maximum in most of the tests, FH hydrolysates were chosen for further analysis to observe the effects of digestive enzymes (pepsin and trypsin) on the antioxidant activities of these bioactive peptides.

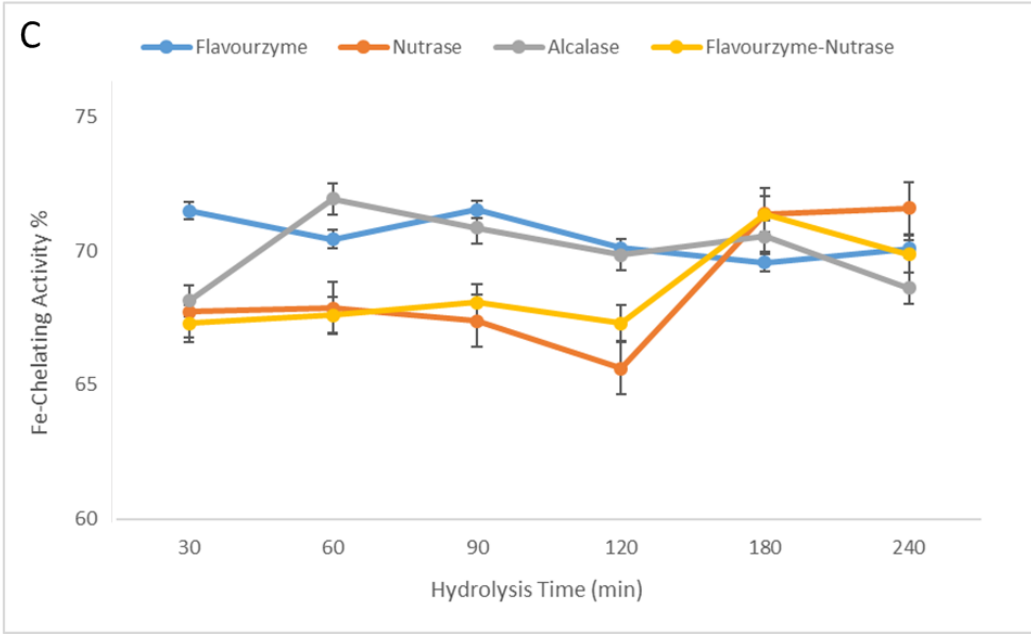
In vitro gastric and small intestine digestions

The DPPH radical scavenging activities for hydrolysates produced after digestion with pepsin are presented in Figure 3.3A and indicated that the DPPH radical scavenging activity increased after the initiation of pepsin hydrolysis and exhibited the maximum DPPH radical scavenging activity (93.53%) after 60 min of pepsin digestion. The DPPH radical scavenging activity decreased after adding trypsin to the reactions (after 120 min of hydrolysis).

The superoxide radical scavenging activity of digested hydrolysates increased sharply in the first 30 min of incubation and then remained almost constant till 120 min. After adding trypsin to the reactions, the superoxide radical scavenging activity decreased gradually (Figure 3.3B). For Fe^{2+} chelating activity of the digested peptides (Figure 3.3C), the effect of pepsin digestion appeared clearly at the beginning of digestion where the Fe^{2+}chelating activity was maximum after 1h and then decreased gradually. However, the reducing power of hydrolysates digested by pepsin and trypsin enzymes was very similar (Figure 3.3D). long digestion time did not decrease the reducing activity but rather increased gradually over time, with the reducing activity maximum at 6 h. In general, the antioxidant assay results presented in Figure 3.3 showed that the best antioxidant activity was observed during pepsin digestion at 30, 60, and 120 min, whereas the antioxidant activities were negatively affected by trypsin digestion.


A
Flavourzyme
Nutrase
Alcalase
Flavourzyme-Nutrase
DPPH- Scavenging activity %
80
75
70
65
60
55
50
45
40
35
30
Hydrolysis Time (min)
30
60
90
120
180
240


B
Flavourzyme
Nutrase
Alcalase
Flavourzyme-Nutrase
Superoxide Radical Scavenging Activity %
50
45
40
35
30
25
20
15
10
30
60
90
120
180
240
Hydrolysis Time (min)



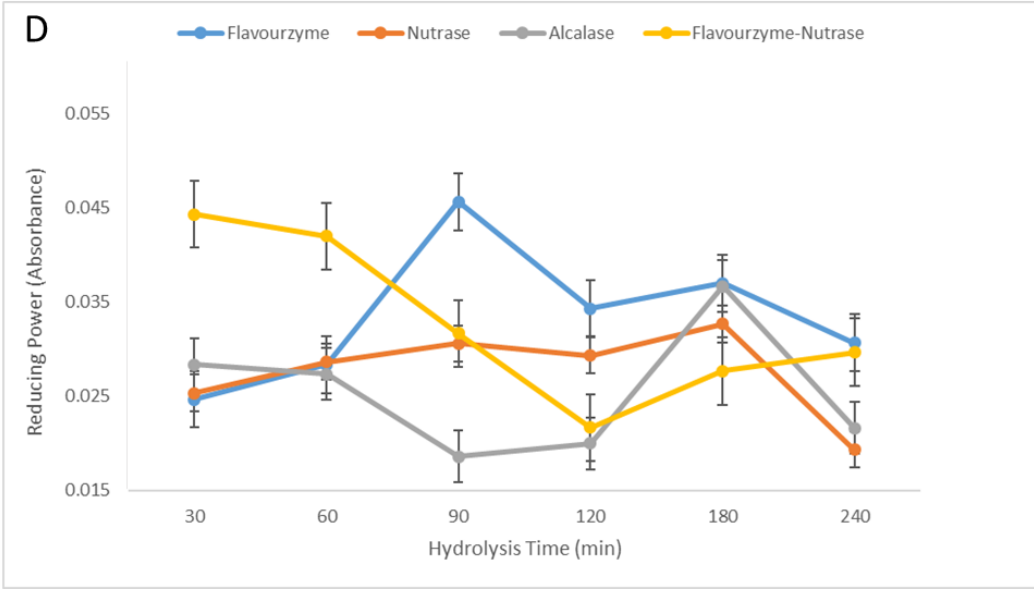
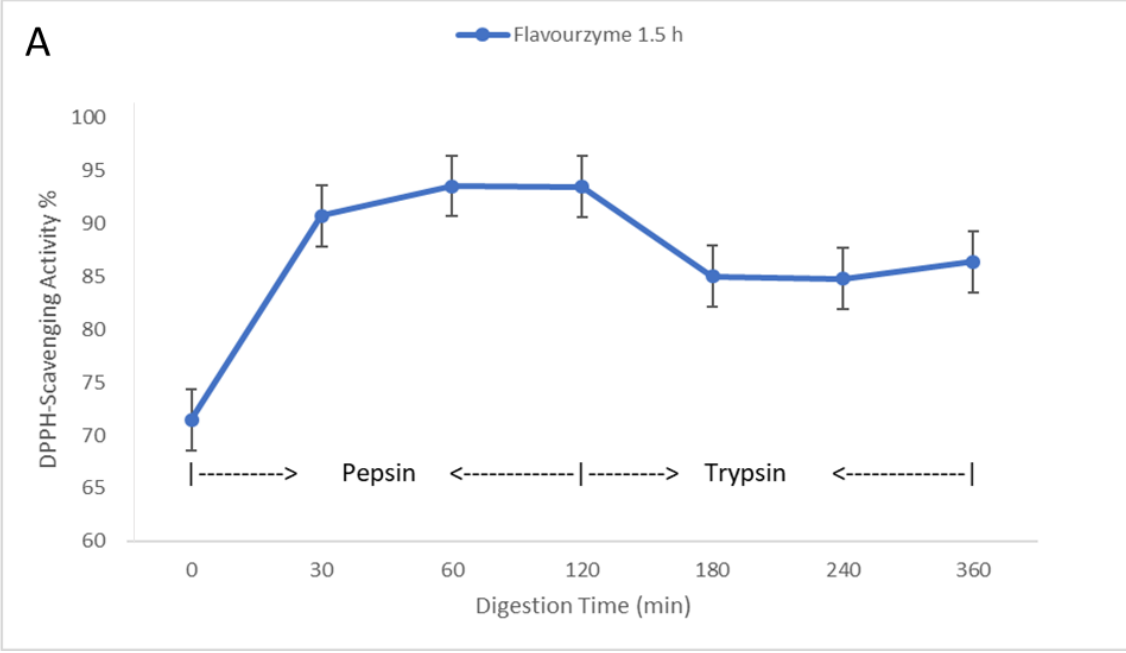
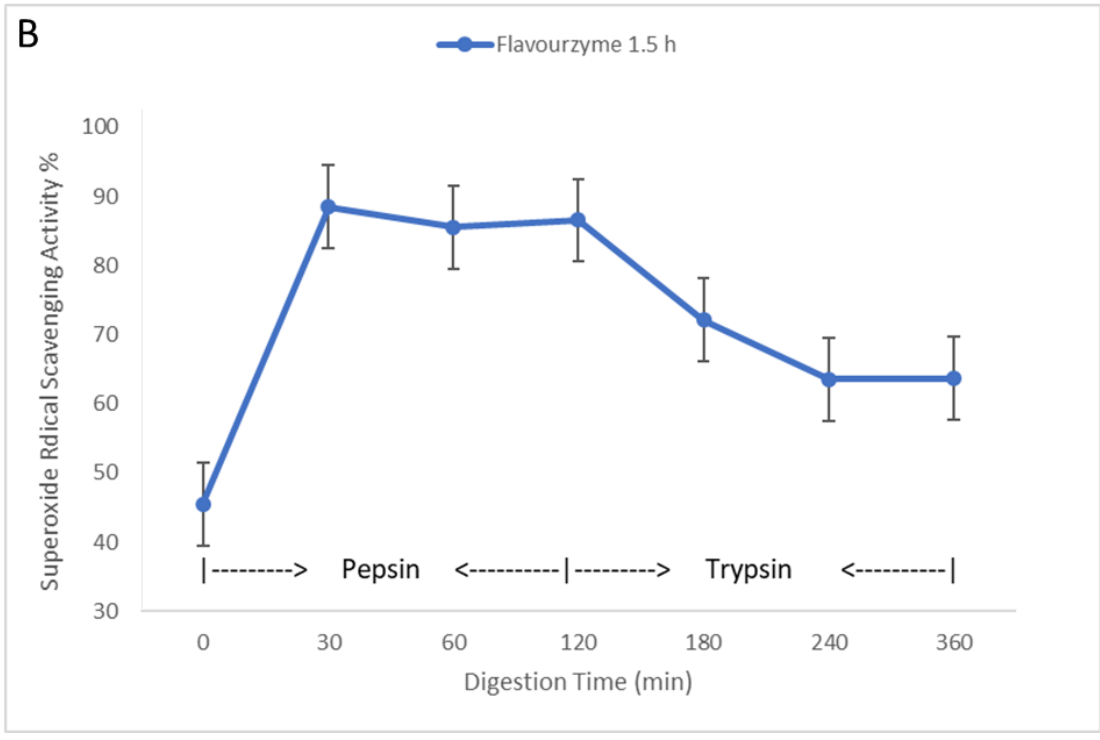


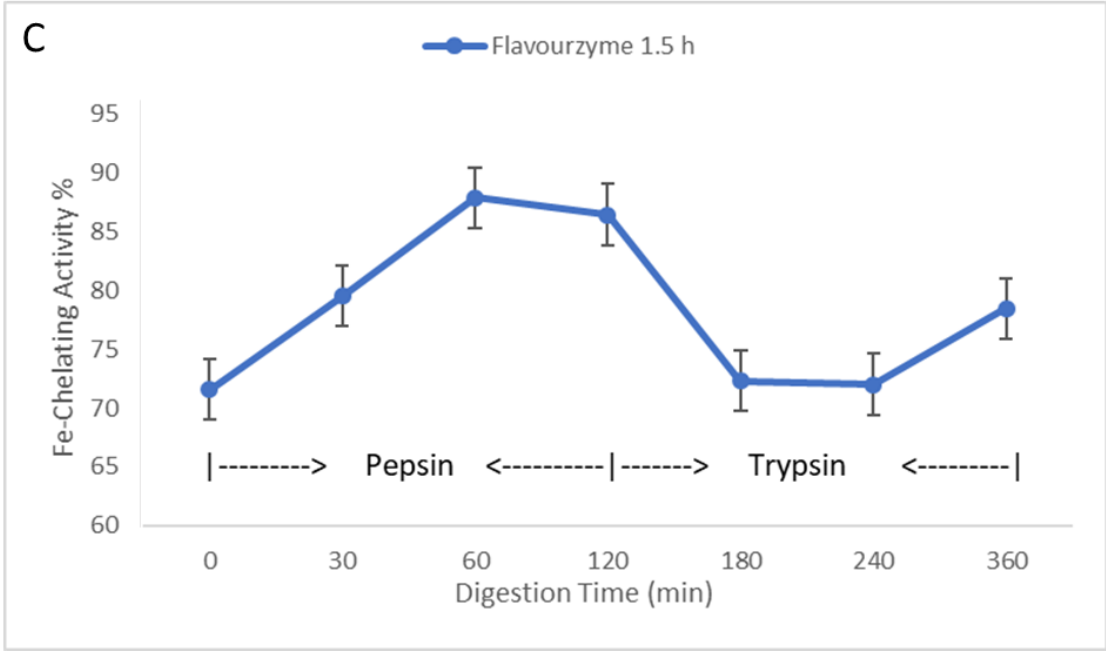
Figure 3.2 A) DPPH radical scavenging activity (% scavenging at 1.0 mg/mL); B) superoxide radical scavenging activity (% scavenging at 0.5 mg/mL); C) ferrous ion chelating activity at 1 mg/mL concentration; and D) reducing power (absorbance at 1 mg/mL) of the camel milk whey protein hydrolyzed by four different proteases for different hydrolysis times (0.5-4h).

Digesta molecular weight (Mw)

The molecular weight (Mw) of FH hydrolysate before, during and after digestion by pepsin and trypsin is shown in (Figure 3.4). Size exclusion-high performance liquid chromatography (SE-HPLC) was used to determine the Mw of the FH digesta. A steady reduction in the size of the produced peptides during digestion was observed (Figure 3.4).




B
Flavourzyme 1.5 h
Superoxide Rdical Scavenging Activity %
100
90
80
70
60
50
40
30
|---------> Pepsin <----------|---------> Trypsin <----------|
0
30
60
120
180
240
360
Digestion Time (min)


C
Flavourzyme 1.5 h
Fe-Chelating Activity %
95
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|---------> Pepsin <----------|-------> Trypsin <---------|
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Digestion Time (min)

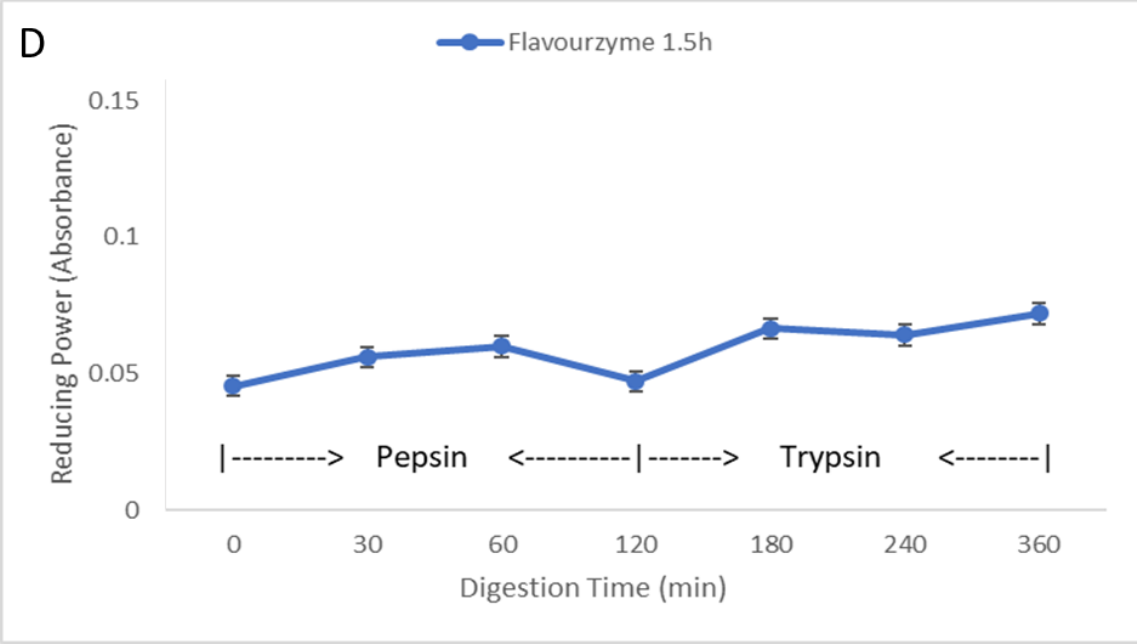

Figure 3.3:A) DPPH free radical scavenging activity (% scavenging at 1.0 mg/mL), B) superoxide radical scavenging activity (% scavenging at 0.5 mg/mL), C) Ferrous ion chelating activity (% at 1.0 mg/mL), D) Reducing power (absorbance at 1.0 mg/mL) of flavourzyme hydrolysate at 1.5 h (FH) during pepsin and trypsin digestion.

The size exclusion chromatograms of undigested and digested samples were divided into 4 groups according to their MW. Group I represent the peptides with very large size (Mw> 50 KDa), group II represents peptides with large size (10KDa<Mw<50KDa), group III represents peptides with medium size (2KDa<Mw<10KDa), and group IV represents peptides with small size (Mw<2KDa).

The size exclusion chromatogram of undigested FH hydrolysates was characterized by one major peak corresponding to the Mw of 16.80KDa, three small broad peaks of Mw 710.80, 77.66, and 62.53 KDa, and some very small peaks of Mw between 9.11 to 1.14 KDa. During the pepsin digestion (i.e., after 30, 60, and 120 min of digestion time), the peaks for the very large size peptides (Mw> 50 KDa) as well as the peptides with Mw 1.14 KDa completely disappeared. The

peptic hydrolysates were characterized by one sharp main peak of Mw (6.83KDa) and numerous peaks and shoulder peaks ranging from 12.88 to 2.18 KDa. Moreover, the pepsin hydrolysis caused an increase in medium-sized peptides with Mw between 2 and 10 KDa. Although the trypsin digestion (i.e., after 180, 240, and 360 min of digestion time) caused the elimination of the peaks for MW higher than 10 KDa (Mw >10KDa), it reduced but did not eliminate the peaks of medium size peptides. Moreover, the trypsin hydrolysis led to the formation of small peptides (Mw<2KDa) and shifted the main peak to smaller Mw peptides. The tryptic hydrolysates were characterized by one sharp main peak for Mw (1.16 KDa) and the other three peaks for approximately 8.37, 4.28, and 0.66 KDa peptides (Figure 3.4).

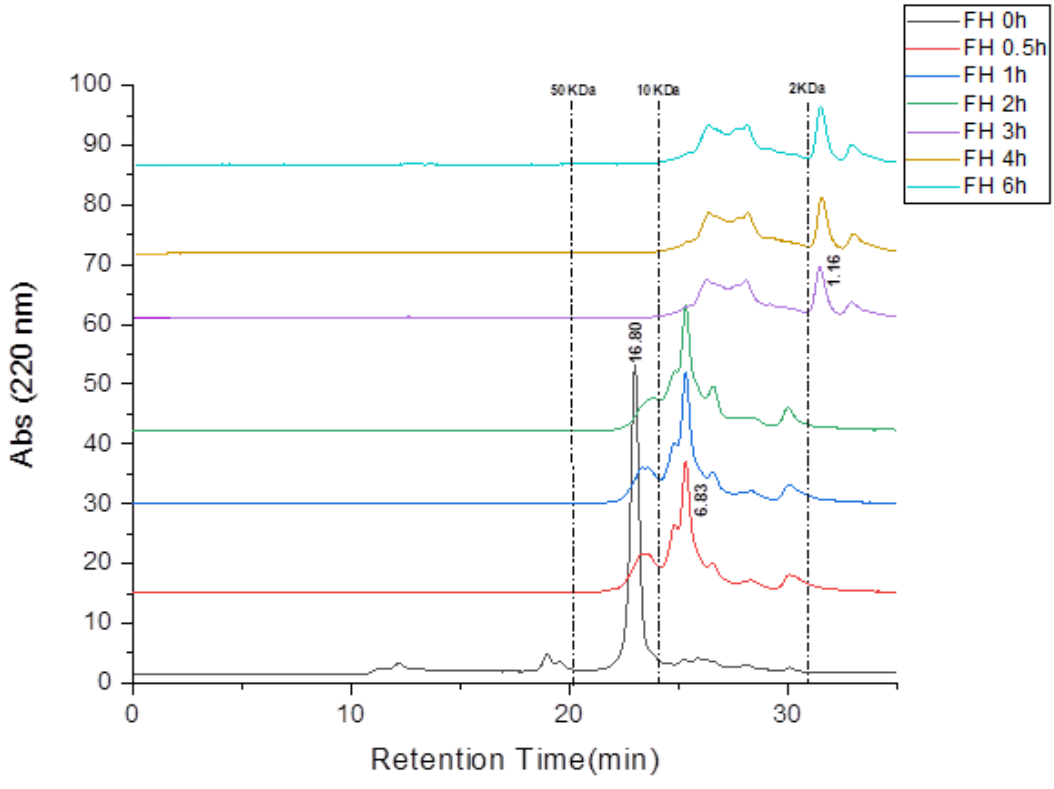

Figure 3.4:Molecular weights of the flavourzyme hydrolysate (FH) before and after digestion with flow rate at 0.1 ml/min.

3.4. Discussion

Several methods are available to estimate the degree of hydrolysis and different approaches are required to determine the DH% of different products. In this study, we used a method that keeps the small Mw peptides (digested peptide products) soluble and causes the large peptides (undigested) to precipitate in the presence of the precipitating agent trichloroacetic acid (TCA). Addition of TCA causes partial or total precipitation of non-hydrolyzed proteins and high molecular-mass peptides [168]. As both an exo- and endopeptidase, flavourzyme achieved the highest degree of hydrolysis. Therefore, flavourzyme has been commercially used for food protein hydrolysis to produce free amino acids and short peptides [174] . These results agree with the barley protein hydrolysates reported earlier [173]. Similar findings have been reported by Xia and other (2012) [175] for glutelin hydrolysates and stated that the flavourzyme hydrolysates had the highest degree of hydrolysis among all other hydrolysates studied. In the TCA method, free amino acids and small peptides remain soluble and stay in the supernatant. However, large peptides will be insoluble after the addition of TCA, and this most likely contributed to the lower degree of hydrolysis [168].

The DPPH free radical scavenging has commonly been utilized as a standard detection method for antioxidant activity. DPPH has a strong scavenging power which entails transferring the hydrogen atom to the free radical to stop the oxidation of the cells and tissues in the body [176]. The ferric reducing antioxidant test was used to determine the reducing power of CMWP hydrolysates by exploiting the capacity of antioxidant substances to reduce Fe^{3+} to Fe^{2+} in a redox-linked colorimetric reaction [173]. Flavourzyme hydrolysate (FH) at 90 min of hydrolysis achieved the maximum DPPH free radical scavenging activity and this may be due to its high content of hydrophobic amino acids which would lead to an increased in surface hydrophobicity (Figure S1;

Appendices). This result is comparable with that of a whey protein hydrolysate [161]. The superoxide radical scavenging activity results have been found to be comparable to camel and bovine whey protein hydrolysates [177] , and the results agree with camel milk casein hydrolysate and whey protein hydrolysate [178]. Amino acid compositions in the bioactive peptides determine the antioxidant activities of peptides with amino acids with ring structures (i.e., imidazole, indole, benzene, and phenol rings such as found in His, Pro, Phe, and Tyr, respectively) providing the best antioxidant activities because the rings act as significant proton and hydrogen donors to free radicals[86]. Cys and Met have strong antioxidant activity because of their ability to extract H+ from S-H groups[177] . The above-mentioned amino acids (i.e., His, Pro, Phe, Tyr, Cys, and Met) comprise about 20% of the total amino acids in FH (the sample before pepsin digestion (FH 0h)). Flavourzyme may work on certain peptides bonds and lead to the release of specific sequences of peptides with superoxide and DPPH scavenging activities [173].

The ferrous chelating activity of bio-peptides is exhibited by two main mechanisms: 1) structurally, entrapping of Fe^{2+} via a specific structure like a "cage structure" and 2) electrostatically binding of Fe^{2+} through charged amino acids. Limited enzymatic hydrolysis of WPCM may transform the structure of hydrolysates (cage structure) to a more adaptable and more amphoteric structure, leading to increase potentials for Fe^{2+} entrapping[179]. Moreover, the enzymatic hydrolysis could expose the charged amino acids (i.e., amino acids having the capacity to binding metal) [173], whereas, extensive hydrolysis can promote loss of the "cage structure" leading to decreased Fe^{2+} chelating activity [179]. These results support the hypothesis that decreases or increase in reducing activities of different hydrolysates may be associated with the changes in amino acid composition. Thus, the exposed high polar or charged amino acids (i.e., "electron-dense amino acids" for example, Glu, Asp, Met, Cys, His, Lys, and Tyr) during the first 90 min of FH hydrolysates could be the reason for the increased reducing power [180].

The results of pepsin and trypsin digestion suggested that specific peptide lengths correlate with antioxidant activity. Pepsin is a non-specific endopeptidase, whereas trypsin is a very specific endopeptidase that cleaves the peptide bond between Arg and Lys [181]. The molecular weight of digesta showed that the peptic hydrolysis produced medium sized peptides that have the highest antioxidant activities. These results supported the theory that there is a relationship between peptide size and antioxidant activity. The pepsin digestion of the sample, which was already hydrolyzed by flavourzyme for 90 min, may help in achieving the optimum peptide size with the highest antioxidant activity. However, further hydrolysis with trypsin and increasing incubation time convert the medium-sized peptides to short peptides and decreased the antioxidant activities. Similar findings have been reported by Peng and other (2009) [161] for whey protein hydrolysates where the authors stated that the medium-sized peptides had the best antioxidant activities. Moreover, a number of studies investigated the antioxidant activities of casein, whey protein, mackerel fish, barley, and egg yolk proteins and confirmed that the medium- and low-medium peptide sizes were the peptide with the highest antioxidant activities [182,183].

The mild denaturation or "molten globule state" in the early stages of enzymatic hydrolysis for native whey protein (mainly α-lactalbumin as a globular protein) increases surface hydrophobicity by exposing the hidden hydrophobic amino acids [184]. These conformational changes and re-arrangement of three-dimensional structures after a brief flavourzyme hydrolysis may encapsulate the hydrophilic amino acids inside as a core with the hydrophobic amino acids acting as a cover (i.e., the hydrophobic surface covered the hydrophilic core) [185] (Figure S1 and Figure S2; Appendices). Even though the aromatic amino acids (Phe and Tyr) are strongly hydrophobic, some of them stay at the surface of protein because of their bulky structure [186,187]. These aromatic amino acids could also participate in the surface hydrophobicity of a molecule [185,173]. Pepsin may be a

non-specific endopeptidase, but it effectively splits hydrophobic peptide bonds preferably at aromatic amino acid sites [181].

The pepsin and trypsin digestion of FH hydrolysates cleaved the peptide bonds and changed the conformational structures and appeared to reveal and unfold the buried hydrophilic side chains. Similarly, Bamdad and others (2011) [173] reported the effects of pepsin, flavourzyme, and alcalase hydrolysis on barley protein by decreasing their surface hydrophobicity. The enzymatic hydrolysis causes the production of charged amino acids and short peptides, thus increase hydrophilicity and decrease surface hydrophobicity [173,188].

The pH of the tested samples was maintained at pH 7 to keep them at states higher than the isoelectric point (pI) of most of the proteins, peptides, and amino acids. There is a strong co-relationship between the degree of hydrolysis by enzymatic digestion and the surface charge. Most of the polar amino acids are charged amino acids like Glu, Asp, Arg, and Lys (strong hydrophilic amino acids) and are usually present on the protein surface [189]. The increase of exposed charged amino acids and poly peptides due to digestion could be responsible for the increased negative charge of the digesta[188] as cleavage of peptide bonds frees amino and carboxylic groups. The carboxylate ions (COO-) will then be separated from carboxylic groups (COOH) to cause the surface charge to become negative [190]. There is also a clear inverse relationship between hydrophobicity and electronegativity of the surface. The charged short-peptides and free amino acids were released and exposed by extensive enzymatic digestion which decrease the hydrophobicity and increase the electronegativity of the whey protein hydrolysate's surface. A similar inverse relationship was also observed between hydrophobicity and electronegativity of the casein protein hydrolysates surfaces upon being hydrolyzed by alcalase [191].

Li-Jun and others (2008) [169] reported that the enzymatic hydrolysis of whey protein reduced the size of the peptides. There is a positive co-relationship between digestion progressions of casein and production of smaller size peptides [188]. The antioxidant activities of peptides are also affected by their molecular weight, an important physical property [86]. The size exclusion chromatogram digesta results confirmed the relationship between size and antioxidant properties of peptides. Several studies have demonstrated the relationship between peptide's size and antioxidant properties. The medium and low-medium size bio-peptides usually exhibited the highest antioxidant activities [161]. A more useful measurement would have been to isolate individual peptides, assess each for its antioxidant properties and determine its individual amino acid composition, but this was not done. The results obtained however suggest that peptide molecular weight is most likely a determining factor for antioxidant activities of peptides after pepsin and trypsin digestion.

3.5. Conclusion

A preliminary assessment of the antioxidant properties of camel milk whey protein hydrolysates indicates that they are not fundamentally different from those derived from bovine milk. Among the studied proteases, pepsin can digest FH hydrolysates effectively to get peptides with suitably high antioxidant activity, whereas antioxidant activities of peptides were negatively affected by trypsin digestion. This result suggested that the molecular weight (MW) is most likely the determining factor for the antioxidant activities of peptides. These results support the hypothesis that hydrolysates of camel milk whey protein have a high potential to be used as a natural, safe, and efficient antioxidant agent for pharmaceutical implementation or food additives.

Chapter 4. **Identification of peptides from camel milk that inhibit starch digestion**

- Advanced revision of this chapter has been prepared as a manuscript for publication.

4.1. Introduction

Starch is the storage polysaccharide in seeds of many plant crops including legumes and cereals, and it is the main constituent of many food products consumed widely in the world [192]. Starch is the only plant polysaccharides that is hydrolyzed by human intestinal enzymes and provides 45-65% of the daily dietary energy for most of the people worldwide [193]. Starch consists of 74%-82% amylopectin and 18%-26% amylose depending on the variety of the source plants [194,68]. Based on its digestibility, starch has been classified as nonglycemic and glycemic starch [193,195]. Glycemic starch includes rapidly digestible starch (RDS) and slowly digestible starch (SDS); non-glycemic starch or resistant starch (RS) is not digested in the small intestine but fermented in the large intestine [194].

In the gastrointestinal tract, there are three stages of starch digestion and utilization: the intraluminal stage which involve digestion by salivary and pancreatic α-amylases, the brush border stage which involve maltase/glucoamylase (MGAM, EC 3.2.1.20/3.2.1.3) and sucrase/isomaltase (SIM, EC 3.2.1.48/3.2.1.10) as the main brush border enzymes, and finally the glucose absorption [72]. Digestion of consumed glycemic starch especially the RDS leads to fast rise of blood glucose levels (hyperglycemia). There is a clear relationship between postprandial hyperglycemia and diet-related health problems like diabetes and obesity [196]. Recently, consumers and researchers are looking negatively upon RDS due to immediate and rapid glucose liberation. On the other hand, slowly digestible starch (SDS) liberate glucose slowly and is desired and recognized healthier than

the RDS [196]. The most useful therapy for diet-related health problems is to reach the optimal level of postprandial blood glucose [197].

The ratio of amylopectin to amylose, crystallinity, porosity, surface area, integrity degree, and food matrix interaction with starch directly affect starch digestibility[68]. The incorporation of other food matrices with starch leads to notable changes in the chemical, physical, and nutritional characteristics of starch that influence the digestibility through inactivating the targeted responsible digestive enzyme [198]. Novel helical complexes like V-type crystalline will be produced when free fatty acids or/and monoglycerides interact with amylose, resulting in the crystalline amylose becoming more resistant to digestion [199]. Interactions between starch and phenolic compounds also decrease starch digestibility by several mechanisms including inhibition of pancreatic α-amylase and brush border enzyme, and enhancing amylose crystallinity [200,201]. Delaying carbohydrate digestion is indispensable for the most beneficial treatment of type2-diabetes [202].

In nature, the proteins in wheat and other grains physically surround the granules of starch, which prevents digestive enzyme access [203]. Many studies have demonstrated that protein surrounding the starch strongly alter the digestion process of starch [204]. However, some protein and bioactive peptides derived from protein are also inhibitors of starch digestion[49,193]. The main potential approach for these peptides is inhibition of the enzymes responsible for starch digestion, such as α-amylase, α-glucosidase, and maltase/glucoamylase [197], through binding to the target enzyme's active sites (catalytic sites or/and substrate binding sites) via hydrophobic interactions [197,202].

Milk is recognized as one of the main natural sources of beneficial bioactive composites. Bioactive peptides derived from milk are generated by hydrolysis of proteins *in vivo* and/or *ex vivo*, through digestive enzymes, microbial enzymes, and microbial fermentation [3]. Milk derived bioactive

peptides exhibit antihyperglycemic property that reduce the glucose level in the blood. Most of the studies with milk are limited to the study of bovine milk, and only a handful of studies have investigated the bioactive peptides from camel milk. Most of the research on camel milk has focused only on raw milk, casein, and fat. Camel milk whey protein has been always overlooked [21].

Numerous *in silico*, *in vitro*, *in vivo*, and clinical studies have demonstrated that peptides from milk product hydrolysates are a good source of antihyperglycemic agents and bioactive peptides with starch digestion inhibitory activity like α-amylase inhibitors [3,205] . These studies used milk protein hydrolysates [121], casein hydrolysates [122], and whey protein hydrolysates [123].

Despite the ability of antihyperglycemic activity of whey protein and whey protein hydrolysates, most of studies used the whey protein and its hydrolysates as is without determining the responsible peptides. Most of the studies that illustrated the starch digestion inhibitory activity of synthetic peptides depended on an *in-silico* analysis and showed the potential active fragments from whey protein and other dietary protein [206,207]. Camel milk whey proteins consist of a high amount of hydrophobic amino acids and contain most of the essential and non-essential amino acids with high concentration of F, V, L, K, E, and P [46,21]. These intrinsic characteristics of camel milk whey protein make it a promising candidate to produce bioactive peptides with starch digestion inhibitory activity.

The aims of this study therefore were: 1) to assess the effect of enzymatic hydrolysis (flavourzyme) on the starch digestion inhibition activities of camel milk whey protein, 2) to determine the effect of amino acid charge and/or hydrophobicity on the starch digestion inhibition activities through purification of the peptides by cation exchange chromatography (CEX) and hydrophobic interaction chromatography (HIC), and 3) to identify the sequences for the most active peptides.

4.2. Materials and methods

Isolation of cheese whey.

Cheese whey was separated by treatment of reconstituted lyophilized milk from Bactrian camels (BC) (Inner Mongolia Agricultural University, China) or bovine milk with camel chymosin (Chr. Hansen, Bayswater, Australia) or bovine chymosin (rennet), respectively. Briefly, skim camel milk solution was reconstituted by dissolving defatted camel milk powder into water at a powder to water ratio of 1:10 (w/v) with vigorous stirring (1000 rpm, 23˚C) for 2h. The reconstituted milk was then heated to 37˚C and 1mL camel chymosin was added to 1L milk, followed by incubation at 37℃ for 60min. Precipitated proteins were removed from the supernatant containing whey protein by centrifugation at 5,000 ×g for 60min at 4˚C. The latter step was repeated 3 times, and the supernatant was lyophilized and stored at -20˚C until further analysis.

Hydrolysis of whey protein.

Whey proteins were hydrolyzed by protease from *Aspergillus oryzae* (Flavourzyme) (Sigma, Canada) **(**500U/g; EC: 232-752-2). A 10% (w/v) whey solution was prepared, and the pH value for solution was adjusted to 6.0 using 0.1M HCL. Flavourzyme was added at 0.05% (v/v) and the mixture was agitated with glass beads at 50˚C for 24h to hydrolyze whey proteins. The hydrolysis reaction was stopped by heating to 95ºC for 5min, then the hydrolysates were lyophilized and stored at −20°C for further analysis. The hydrolysis was conducted in triplicate.

In vitro digestibility of starch and starch-peptide mixture

Starch (7.5mg; potato starch, Sigma, Canada) or peptide-starch mixture (2.5mg peptide: 7.5mg starch) were suspended in 1mL of water, heated for 10min at 85℃ to gelatinize the starch, and incubated at 37℃ for 16h. Digestion was carried out by adding 0.5mg of pepsin (250U/mg, Sigma,

Canada) and incubation at pH 2.0 and 37℃ with agitation at 200rpm for 30min. The pH of the digesta was adjusted to pH 6.0 with 2M NaOH prior to addition of brush border enzymes from the rat intestinal mucosa, and porcine pancreatic enzymes. In brief, 1ml of 50mM sodium maleate buffer pH 6.0 containing 0.07g pancreatin from porcine pancreas enzymes (Sigma, USA; 45U/mg lipase, 42U/mg amylase, and 3.0U/mg protease) and 10g/L rat small intestinal enzyme (Sigma, USA) was added to 1ml of resulting digesta solution. After adding ~ 5 glass beads (5mm diameter), the reaction mixture was incubated at 37℃ and pH 6 for 4h with agitation at 200rpm. The digestion process was stopped by heating to 95℃ for 4min. The samples were cooled on ice and centrifuged at 5, 000 ×g for 5min at 4˚C. The glucose concentration for samples and controls was measured with the D-glucose (GOPOD-format) kit (Megazyme, Bary, Ireland) (Figure 4.1).

Hydrophobic interaction chromatography (HIC)

Camel milk whey hydrolysates were fractionated by hydrophobic interaction chromatography (HIC) on an Octyl Sepharose CL-4B column (1.5cm × 15cm, Octyl Sepharose CL-4B, GE Healthcare, Chicago, IL) that was linked to a UV detector (220nm). Freeze-dried camel milk whey hydrolysates were dissolved in distilled water to a concentration of 1g/L and the pH was adjusted to 6.0. Of this solution, 250mL were loaded on the column. The column was washed with 250mL distilled water (pH 6) and eluted with 250mL 5% isopropanol in water. The fractions were pooled based on the 220 and 280 nm absorbance, freeze-dried, and analyzed by starch digestibility assay as described above (Figure 4.1).

Cation exchange chromatography (CEX)

Camel milk whey hydrolysates were fractionated by cation exchange chromatography (CEX) on a 1.5cm × 15cm, SP- Sepharose fast flow column (GE Healthcare, Chicago, IL). Freeze-dried

camel milk whey hydrolysates were dissolved in distilled water to a concentration of 1g/L and the pH was adjusted to 7.0. Of this solution, 250mL were loaded on the column and the column was washed with distilled water (pH 7). The column was eluted with a linear gradient of 0 to 2M NaCl in water and the fractionation was monitored by measuring the absorption at 220nm. The fractions were pooled based on peaks, then part of each pooled fraction was freeze-dried, and analyzed for the starch digestibility assay as described above.

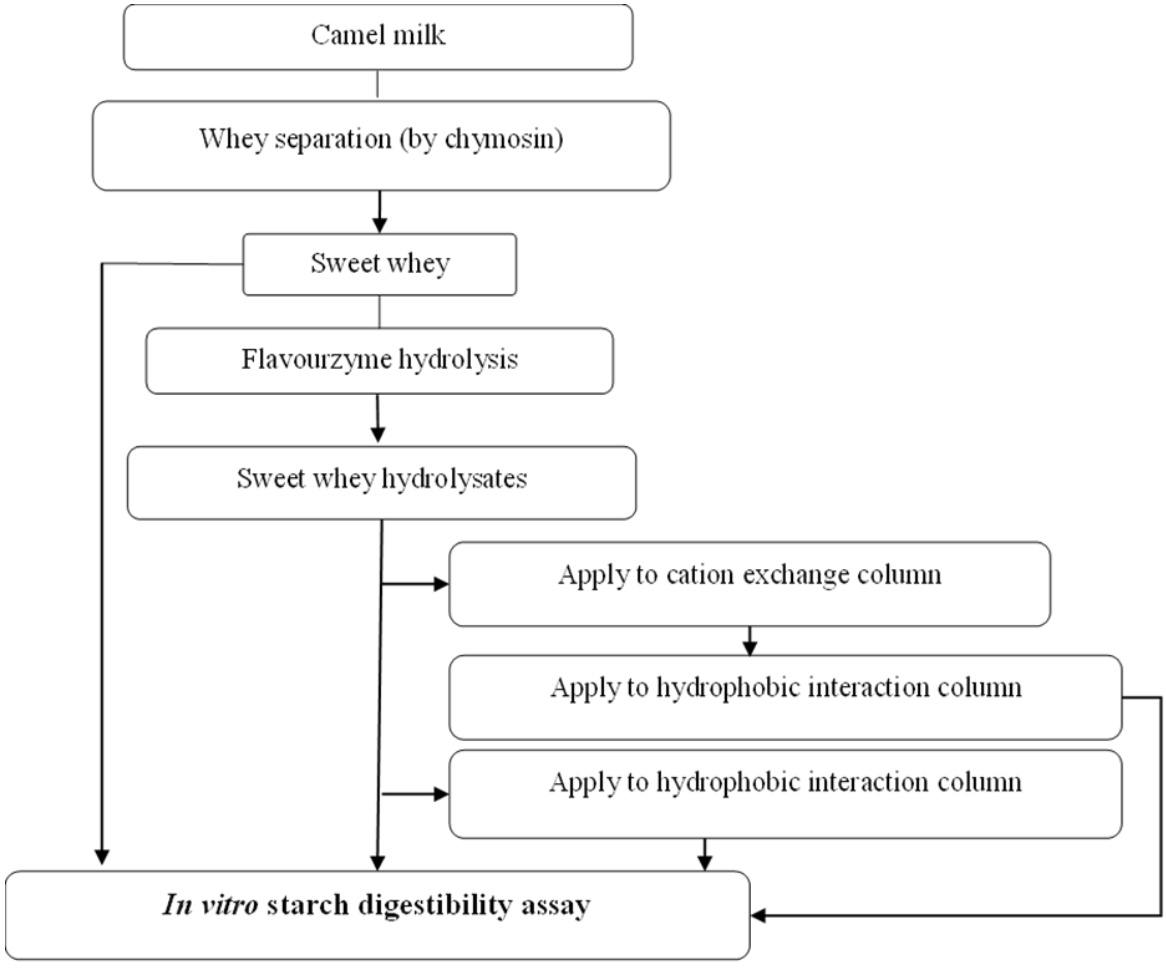

Figure 4.1:Flow diagram for the isolation of bioactive peptide from camel milk whey protein.

Fraction (F1) from the cation exchange column (Figure 4.4) was further purified and sub-fractionated by hydrophobic interaction chromatography (HIC) as described above. The column

was washed with 0.1% trifluoroacetic acid (TFA) and eluted with 5% isopropanol in 0.1% TFA. The fractions were pooled based on peaks, freeze-dried, and analyzed for the starch digestibility assay as described above (Figure 4.1).

Size exclusion chromatography (SEC)

The size of protein or peptides resulted from camel whey, camel whey hydrolysate, selected fraction 1 from hydrophobic interaction chromatogram, and selected subfractions from cation exchange chromatogram and hydrophobic interaction chromatogram were determined by size exclusion chromatography on a Superdex peptide 10/300 GL column (30cm × 10mm, 8.6µm, GE Healthcare Bio-Sciences, Uppsala, Sweden) and ZORBAX PSM 60 HPLC column (6.2 x 250mm, 5µm, Agilent Zorbax, Santa Clara, California, United States) respectively, as a back to back system. Size determinations were carried out on an Agilent 1200 HPLC system coupled to multiple wavelength (220 and 280 nm) and refractive index detector, at 10 ul injection volume, flow rate 0.2ml/min, and isocratic elution with water as mobile phase for 240 min.

Peptide sequencing

The fractions with the best starch digestion inhibition activity were selected for peptide sequencing. Peptide sequencing by LC-MS/MS was carried out by Alberta Proteomics and Mass Spectrometry Facility in the Department of Biochemistry, Faculty of Medicine & Dentistry, at University of Alberta. Samples were digested with trypsin prior to analysis. Briefly, 50µg of sample was dissolved in 100mM ammonium bicarbonate to a concentration of 1.0g/L, reduced with dithiothreitol and alkylated with iodoacetamide. Samples were then digested overnight with trypsin (2µg, Promega sequencing grade) at 37˚C. After digestion, the pH of the samples was

adjusted to 3-4 with formic acid, dried, dissolved in water + 0.2% formic acid, and desalted (Pierce C18 tips).

The tryptic peptides were resolved and ionized by using nano flow HPLC (Easy-nLC 1000, Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) with an EASY-Spray capillary HPLC column (ES800A, Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey orbitrap spectra using external mass calibration, with a resolution of 35,000 and m/z range of 300–1700. The twelve most intense multiply charged ions were sequentially fragmented by using HCD dissociation and spectra of their fragments were recorded in the orbitrap at a resolution of 17,500. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the database was searched using SEQUEST (Thermo Scientific). Search parameters included a strict false discovery rate (FDR) of 0.01, a relaxed FDR of 0.05, a precursor mass tolerance of 10ppm and a fragment mass tolerance of 0.01Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

Peptide synthesis

Thirty-seven (37) peptide sequences were identified within the most potent HIC fractions and SEC+HIC subfractions by using LC/MS/MS, six short peptides were chosen for peptide synthesis. The selected peptide sequences were synthesized by Canada peptide (Pointe-Claire, Quebec, Canada) with 92.5 – 97.9 % purity. Then, the starch digestibility assay was applied as described above.

Statistical Analysis.

Starch digestibility assay was performed in triplicate biological repeats with three technical repeats, and the results are presented as means ± standard error. To determine the statistical differences between the samples, p-values were calculated using Tukey Pairwise Comparisons at 95% Confidence in Minitab 19 (The differences between the conditions are considered significant if p-value < 0.05).

4.3. Results

Starch digestibility inhibition of camel whey and whey hydrolysates.

The starch digestibility assay was applied for starch alone or mixtures of peptides / proteins and starch in a ratio of 1:3 (peptide: starch). The digestibility assay included addition of pepsin, pancreatic enzymes, and brush border enzymes to mimic the enzymes involved in starch and protein digestion in the digestive tract. The activity of brush border glycosyl hydrolases from rat intestinal mucosa corresponds the activity of human brush border enzymes[208].

Whey and casein inhibited starch digestion by about 10 and 7%, respectively (Table 4.1). Enzymatic hydrolysis of whey and casein with flavourzyme increased the inhibition of starch digestion by whey and casein hydrolysates to about 17 and 11%, respectively (Table 4.1). Hydrolyzed whey consists of 13.6% proteins or peptides while the protein or peptide content in the casein hydrolysate is more than 85%, therefore, any peptides in the whey fraction presumably are more active and subsequent analyses focused on whey hydrolysates.

Purification of Bactrian camel whey hydrolysate.

Peptides were fractionated either by hydrophobic interaction chromatography (HIC), or by cation exchange chromatography (CEX), followed by HIC separation of the most active fractions

Table 4.1 Peptide recovery after chromatography on SP- Sepharose fast flow column (cation exchange chromatogram; CEX) and on Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC), and starch digestibility inhibition of collected fractions (peptides) at the ratio 1:3 (peptide: starch) respectively. The intact and hydrolyzed casein and whey proteins samples were performed in triplicates and fractions were collected in duplicate.

Sample	**Amount of protein or % of protein recovered after chromatography**	**Inhibition of starch digestion**
Camel milk	--	not determined
Casein	--	7.6% ±1.1
Whey	--	10.1% ±0.9
Hydrolyzed casein	--	11.3% ±0.9
Hydrolyzed whey	250mg, corresponding to 34mg whey protein	16.5% ±0.2
Fraction 1 after HIC	32%	26.9% ±0.1
Fraction 1 after CEX	86%	24.1% ±0.1
Fraction 1 after CEX and HIC	45%	32.8% ±0.4
Fraction 2 after CEX and HIC	09%	35.7% ±0.3

(CEX+HIC). Fractions were characterized with respect to the inhibition of starch digestion. Fractionation of whey hydrolysate by HIC resulted in five fractions (Figure 4.2). Among these fractions, fraction 1 (F1) was most inhibitory to starch digestion (Figure 4.3).

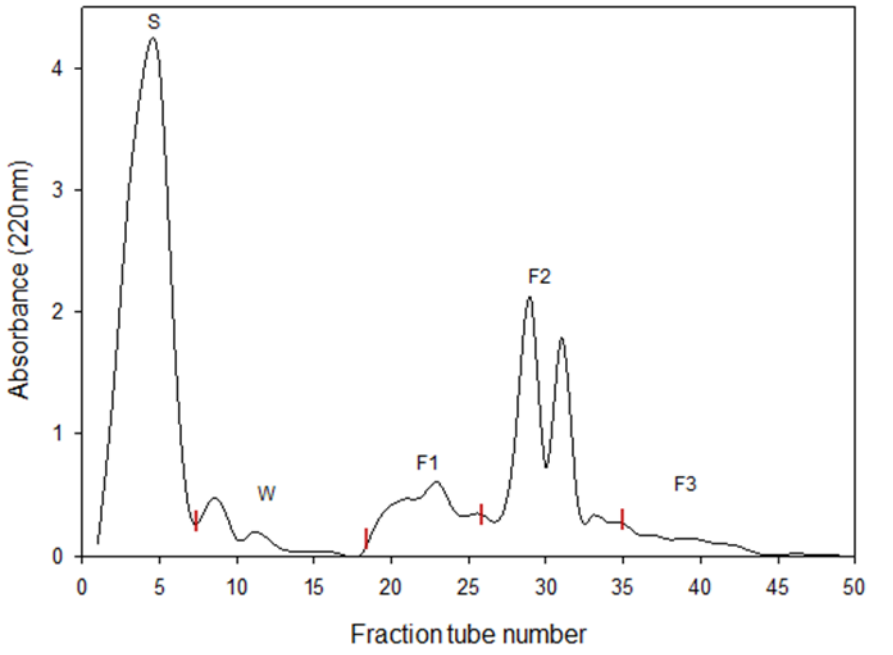

Figure 4.2: Fractionation of flavourzyme hydrolyzed camel whey on Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). Hydrophobic peptides were eluted with 5% isopropanol. However, the volume of every fraction tube is 5mL.

Whey hydrolysate purified by CEX was collected in four fractions (Figure 4.4A). The chromatogram and the inhibition of starch digestion by the fractions are shown in Figure 4.4B and 4.5B, respectively. Peptides eluting in fraction 1 (F1) were further fractionated on a HIC column. Of the fractions eluting from the HIC column, fractions 1 and 2 were most inhibitory to starch digestion and were chosen for peptide sequencing by LC-MS/MS. Fraction 1 eluting from HIC was also sequenced for comparison.

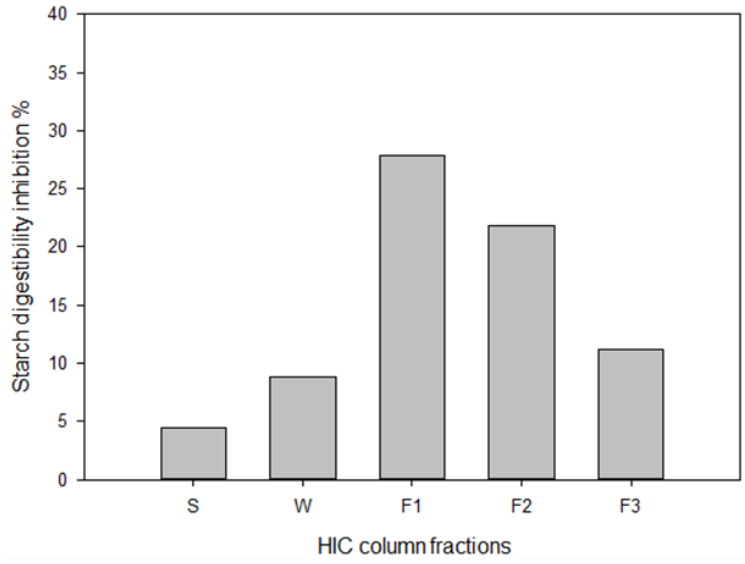

Figure 4.3: Starch digestibility inhibition % of flavourzyme hydrolyzed camel whey fractions resulted after applying on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC).

Determination of peptides size by size exclusion chromatography

The active peptides and fractions were analyzed by SEC to determine the effect of flavourzyme hydrolysis and (HIC) or (CEX and HIC) fractionation on the size and molecular weight of whey protein and whey protein hydrolysates particles to confirm the relationship between molecular weight of the resulted peptides and their starch digestion inhibition activity. Figure 4.6 showed that the flavourzyme hydrolysis of camel whey protein and the fractionation of camel milk protein hydrolysates led to a decrease in the molecular weights of the original protein and protein hydrolysate, respectively. The starch digestion inhibition activity increased with decreasing the molecular weights of the resulting peptides (Figure 4.6).

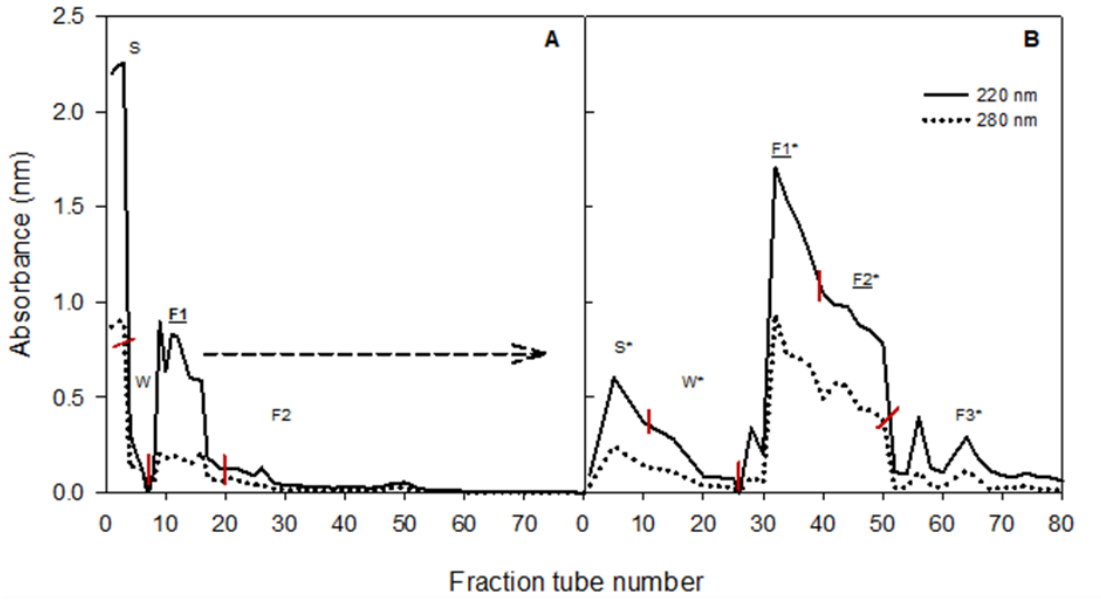

Figure 4.4: **Panel A:** Fractionation of flavourzyme hydrolyzed camel whey sample on SP- Sepharose fast flow (cation exchange chromatogram; CEX). **Panel B:** Fractionation of F1 eluting from CEX on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). However, the volume of every fraction tube is 5mL.

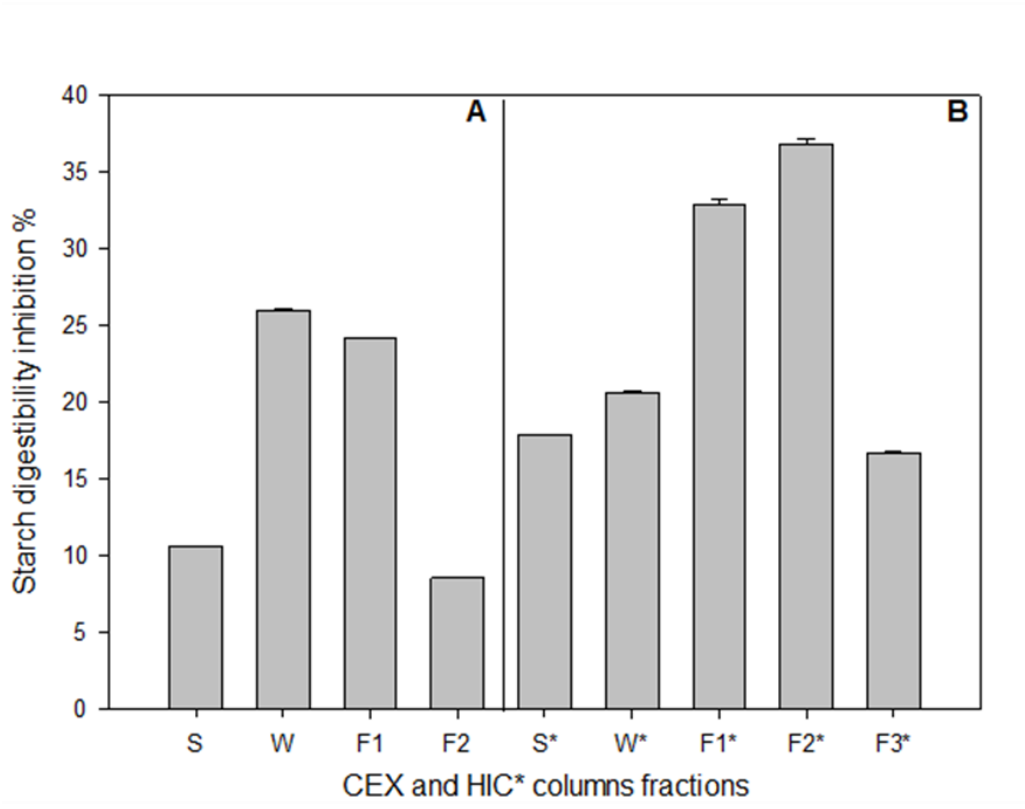

Figure 4.5: Starch digestibility inhibition % of flavourzyme hydrolyzed camel whey. **Panel A.** Fractions after separation on SP- Sepharose fast flow (cation exchange chromatogram; CEX). **Panel B.** Fractions after separation on CEX and subsequent separation of fraction F1 on an Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC).

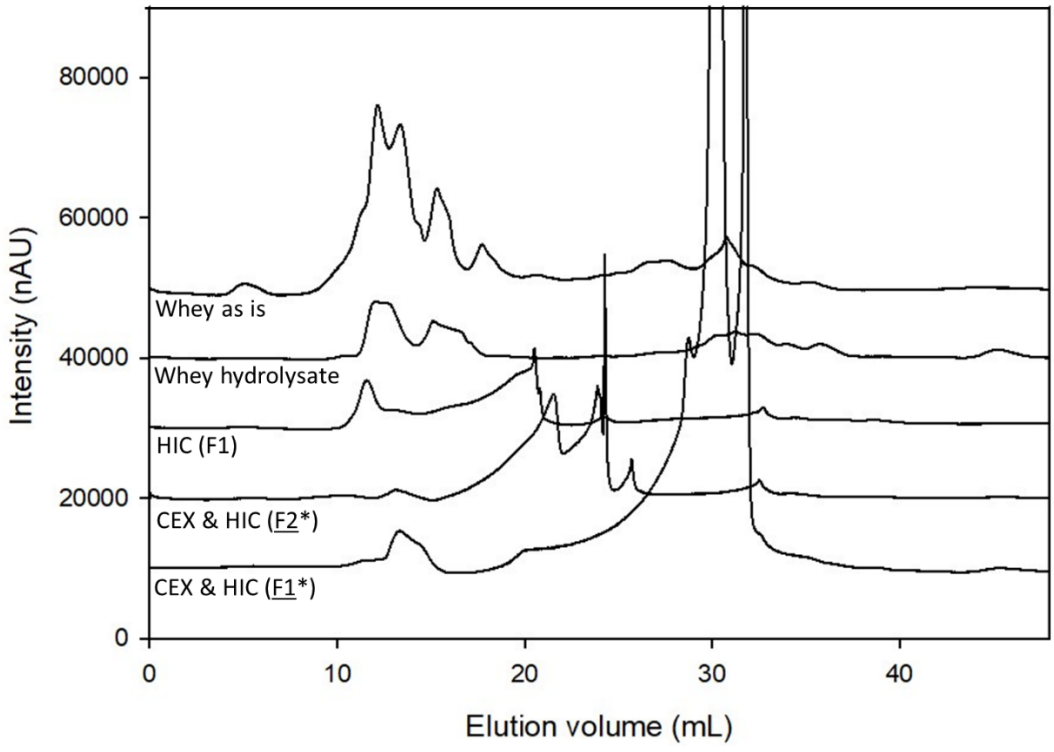

Figure 4.6: Size determination of camel whey, camel whey hydrolysate, selected fraction 1 from hydrophobic interaction chromatogram (HIC (F1)), and selected subfractions from cation exchange chromatogram (CEX) and hydrophobic interaction chromatogram (HIC); (CEX & HIC F2*) and (CEX & HIC F1*), through size exclusion chromatography at 220 nm. The lower and upper exclusion limit is 90000 and 509565, respectively.

Table 4.2**.** Sequences of peptides resulted from camel whey protein hydrolysates after separation on an octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC).

No.	Sequence	RT (min)	Charge	MW (Da)
1	VTMQNLNDR	13.30	2	1090.53
2	**IRDWYQR**[a)]	13.35	3	1036.53
3	**LVPVICHR**	13.47	3	993.57
4	GFSSGSAVVSGGSR	13.54	2	1254.61
5	**LASYLDKVR**	14.64	3	1064.61
6	**YFCDNQETISSK**	14.65	2	1491.64
7	ALEEANADLEVK	16.31	2	1301.66
8	**IRLENEIQTYR**	16.60	3	1434.77
9	**FLEQQNQVLQTK**	16.82	2	1475.79
10	**FASFIDKVR**	17.27	3	1082.60
11	**RHPEYAVSLLLR**	18.90	3	1453.83
12	DAEAWFNEK	19.64	2	1109.49
13	VLDELTLAR	19.79	2	1029.59
14	**EYGLFQINNK**	20.68	2	1225.62
15	WELLQQVNTSTR	22.06	3	1475.75
16	**VVSVLTIQHQDWLTGK**	22.25	3	1824.01
17	**NMFETPFLAR**	23.81	2	1225.60
18	LALDIEIATYR	24.15	2	1277.71
19	**FLEQQNQVLQTKWELLQQVNTSTR**	24.72	4	2932.52
20	SLDLDSIIAEVK	27.38	3	1302.72
21	**VNLFDIPLEVQYVR**	29.23	3	1704.94
22	**LALDVEIATYR**	57.04	2	1263.70

[a)]Sequences that were not identified after fractionation on a cation exchange column are printed in bold and underlined.

Table 4.3Sequences of peptides identified in camel whey protein hydrolysates after separation on SP- Sepharose fast flow column (cation exchange chromatogram; CEX) and on Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC). Peptides were sequenced by LC-MS/MS after trypsin hydrolysis of the fractions unless indicated.

No.	Sequence	RT (min)	Charge	MW (Da)
1	NKYEDEINKR	10.72	3	1308.66
2	VTMQNLNDR	13.25	2	1090.54
3	GFSSGSAVVSGGSR	13.43	2	1254.61
4	YEELQVTAGR	15.32	2	1165.59
5	ALEEANADLEVK	16.20	2	1301.67
6	YEELQITAGR	16.79	2	1179.61
7	WTLLQEQGTK	18.67	2	1203.64
8	DAEAWFNEK	19.41	2	1109.49
9	VLDELTLAR	19.59	2	1029.59
10	GSLGGGFSSGGFSGGSFSR	20.23	2	1707.77
11	WELLQQVNTSTR	21.84	3	1475.75
12	LALDIEIATYR	24.00	2	1277.71
13	SLDLDSIIAEVK	27.17	2	1302.72
14	KKAGVLDYETFTK*	6.35	3	1499.81
15	KHSTKGLGK*	14.76	2	955.57

(*) peptides that were identified in samples in Fraction 2 that were not hydrolyzed with trypsin prior to LC-MS/MS analysis.

Peptide sequences in fractions inhibiting starch digestion.

The derived peptides were sequenced by LC/MS/MS after trypsin digestion. A total of 22 peptides were identified in fraction 1 after hydrophobic column (HIC) separation. Peptide sequences consisted of 7-24 amino acids with molecular weights (Mw) ranging from 994 to 2933Da (Table

4.2). In fractions obtained after fractionation on (CEX and HIC), 13 peptides were identified after trypsin digestion. In addition, 2 peptides were identified in a sample that was analyzed without a trypsin digestion step. The molecular weights of the peptide sequences ranged from 956 to 1708Da, and the peptides contained 8-19 amino acids (Table 4.3).

Inhibition of starch digestion by synthesized peptides

The starch digestibility assay as described above was applied for the selected synthesized peptides sequences to determine their starch digestion inhibition activities. Table 4 illustrated that two sequences that were identified after fractionation on (HIC) and (CEX and HIC); LALDIEIATYR (LR11) and VLDELTLAR (VR9) are as active as the entire fraction. LR11 and VR9 inhibited starch digestion by about 37 and 33%, respectively. However, the remaining peptides are inactive or much less active (Table 4.4).

Table 4.4 Starch digestibility inhibition (%) of synthesized peptides identified in camel whey protein hydrolysates after separation on SP- Sepharose fast flow column (cation exchange chromatogram; CEX) and on Octyl Sepharose CL-4B column (hydrophobic interaction chromatogram; HIC), or just on Octyl Sepharose CL-4B column (HIC) at the ratio 1:3 (peptide: starch) respectively. The peptides synthesized by Canada Peptide company, Pointe-Claire, Quebec, Canada.

Camel Peptide sequences	Protein Access.	Camel Protein	Peptide source (column)	Starch digestibility inhibition%
LALDIEIATYR	S9WX05	Uncharacterized	HIC and CEX+HIC	37.4 ± 1.4^{a}
VLDELTLAR	S9XAP9	Intermediate filament	HIC and CEX+HIC	33.6 ± 2.4^{a}
DAEAWFNEK	S9WUY9	Intermediate filament	HIC and CEX+HIC	5.5 ± 2.1^{b}
WTLLQEQGTK	S9Y6J1	Intermediate filament	CEX+HIC	9.5 ± 1.3^{b}
YEELQVTAGR	S9W9S8	Intermediate filament	CEX+HIC	4.6 ± 1.5^{b}
KHSTKGLGK	S9XLY6	Poly [ADP-ribose] polymerase	CEX+HIC	6.7 ± 1.2^{b}

4.4. Discussion

Protein and peptides can delay starch digestion by inhibition of the enzymes responsible for starch digestion, such as α-amylase, α-glucosidase[120,119,193,49]. This study demonstrates that peptides derived from camel milk whey protein have a starch digestion inhibitory activity. The activity was confirmed by the starch digestibility assay. Purification and fractionation of camel milk whey protein peptides depending on the content of hydrophobic and positively charged amino acids strongly increased the inhibition of starch digestion. For two of the peptides, inhibition of starch digestion was confirmed by assays with pure, chemically synthesized peptides.

Camel milk whey protein is not as well studied as bovine milk whey protein and the biological activities of bioactive peptides derived from camel milk whey protein are not fully explored[162]. In this study the combination of enzymatic hydrolysis, ion exchange chromatography, and/or hydrophobic interaction chromatography allowed fractionation and purification of bioactive peptides with starch digestion inhibitory activity from the Bactrian camel milk whey protein. The combination of several techniques allowed purification of antihyperglycemic peptides from bovine milk whey protein [205].

The *in vitro* and *in vivo* studies reported that the antihyperglycemic activity of whey protein hydrolysates are higher than that of the casein hydrolysates [3,121]. This completely agreed with our results and whey protein was found more effective than casein. However, pepsin-treated bovine α-lactalbumin exhibited the highest antihyperglycemic activity compared to other pepsin-treated whey proteins including bovine serum albumin, β-lactoglobulin, lactoferrin, and whey protein isolate, whereas the β-lactoglobulin showed the lowest antihyperglycemic activity [209]. The α-lactalbumin is the major component of camel milk whey protein constituting about 47.41 %, with no presence of the β-lactoglobulin [34]. Bactrian camel milk whey hydrolysates with 13.6% protein

content showed potential starch digestion inhibition activity higher than casein hydrolysate. The fractionation of the camel milk whey protein hydrolysate on HIC column alone produced a peptide fraction that exhibited about 63% higher starch digestion inhibitory activity than the starting hydrolysate. On the other hand, the peptide fractions obtained from the successive chromatographic separations of the same sample by CEX and HIC increased the starch digestion inhibition activity about 116% more than the original hydrolysate (Table 4.1). The positively charged hydrophobic amino acids could help to improve the dietary intervention for delayed starch digestion.

Among the synthesized peptides, LALDIEIATYR and VLDELTLAR showed the highest starch digestion inhibitory activity, whereas the remaining synthesized peptides had only limited starch digestion inhibitory activity. The most potent peptides, LALDIEIATYR and VLDELTLAR were identified in HIC and CEX and HIC fractions. These two peptides were more effective at starch digestion inhibition than the fractionated camel milk whey protein hydrolysate (Table 4.1 and 4.2). In a previous study, the fractionation of the bovine whey protein isolate and α-lactalbumin hydrolysate through successive chromatographic separation generated peptides with more robust starch digestion inhibitory activity than the original starting hydrolysate [205].

Bioactive peptides that inhibit starch digestion act locally in the gastrointestinal tract [51], and the bioavailability of these peptides is affected by digestive enzymes in the gastrointestinal tract, metabolism, and absorption. Pepsin hydrolysis in the stomach is the first step in food protein digestion and the proteins are then further hydrolysed by the pancreatic proteases trypsin and chymotrypsin, and by brush border peptidases that are expressed after the peptides are transport into the epithelial cells of the small intestine [50]. Brush border enzymes that contribute to peptide hydrolysis include the peptidyl dipeptidase, aminopeptidase N, dipeptidyl aminopeptidase IV,

γ-glutamyltranspeptidase, aminopeptidase A, and carboxypeptidase[50,53,55]. The bioavailability of ingested bioactive peptides depends on their composition and the degree of hydrolysis by the digestive enzymes [51,52,53]. Proteins with high content of proline are resistant to gastric and pancreatic peptidases, and proline-rich peptides are thus most likely to escape the digestion and to reach the intestinal membrane in relatively intact sequence to face the brush border enzymes [53,55]. Some milk-derived bioactive peptides like, IPP [56], VPP [57], and HLPLP [60] have been detected in the plasma of human and animals.

There are numerous studies that confirmed starch digestion inhibition activity for protein, protein hydrolysates and peptides derived from foods like albumin [119], legumes [210], cumin [211], and milk [207,209,212]. Different proteins and their derived peptides differ in their bioactive effect. For example, one of the cumin seed derived peptides "FFRSKLLSDGAAAAKGALLPQYW" showed potent α-amylase inhibition activity around 24.5% [211], whereas KLPGF derived from albumin showed good α-amylase and α-glucosidase inhibition with an IC50 values about 120 and 59 μM [119]. Despite the milk proteins and their hydrolysates showing antihyperglycemic activity, no studies have identified the peptides responsible for this activity [206,207].

The antihyperglycemic activity of whey protein hydrolysates derived from using digestive enzymes like pepsin and trypsin have been confirmed *in vitro* [207,209]. The hexapeptide VAGTWY resulted from trypsin-treated whey protein hydrolysate showed significant decrease in postprandial glucose level in mice with an IC_{50} value about 174 μM [212]. Whey protein hydrolysates prepared by tryptic treatment also produced some antihyperglycemic peptides like VLVLDTDYK, TPEVDDEALEK, IPAVFK, and IPAVF with 424.4, 319.5, 143.0, and 44.7 μM as IC_{50} values [213]. Similarly, Lacroix and Li-Chan [205] fractionated and identified several antihyperglycemic peptides from pepsin-treated α-lactalbumin and whey protein isolate through continuous chromatographic

steps, and the strongest peptides were found to be LKPTPEGDL and LKPTPEGDLEIL with IC50 values 45 and 57 μM, respectively.

Pancreatic amylase, maltase and isomaltase are responsible for hydrolyses of 1,4 and 1,6 glucosidic bond of starch polymers, respectively, and the inhibition of these enzymes will lead to delay starch digestion [72]. The starch digestion inhibitory effect of protein hydrolysates is mainly affected by the amino acid compositions of the resulted peptides. However, the starch digestion inhibitory effects of α-lactalbumin hydrolysates has been reported higher than the hydrolysates of whey protein isolate, bovine serum albumin, β-lactoglobulin, and lactoferrin resulted after pepsin treatment [209].

Conclusion

The prevalence of food-related chronic diseases including diabetes mellitus has increased worldwide. Digestion of consumed glycemic starch especially RDS leads to hyperglycemia. Hyperglycemia for a long-time lead to the development of insulin resistance, and then diabetes mellitus. Delaying carbohydrate digestion is the most acceptable approach as a treatment of type2-diabetes. Camel milk whey protein hydrolysates have a potential inhibitory effect on starch digestion. The successive chromatographic separation aiming to produce positively charged peptides with hydrophobic amino acids was shown to increase the starch digestion inhibitory activity relative to the original starting hydrolysate.

Chapter 5. **Glycomacropeptide from camel milk inhibits the adhesion of enterotoxigenic *Escherichia coli* K88 to porcine cells.**

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5.1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of childhood diarrhea in developing countries and cause traveler's diarrhea. ETEC also cause watery diarrhea in newborn and post-weaning piglets and calves of cows and camels [150,151]. ETEC produce two enterotoxins: heat stable enterotoxin (ST) and heat labile enterotoxin (LT). ETEC adhere to the small intestinal epithelial cells and to the mucosal tissue through glycoprotein receptors of the host cells using specific fimbriae. Colonization of the microvilli and the production of enterotoxins lead to electrolytes imbalance and water loss [153,154]. *E. coli* expressing K88 fimbriae are among the most prevalent strains of ETEC that cause diarrhea in swine [214,153].

The mortality rate in farm animals due to bacterial infections is increasing especially at the weaning stage[215,216]. The use of antibiotics to control ETEC in pig production increases costs, supports the emergence of antibiotic resistant pathogens in animals as well as the transmission of antibiotic resistance to human pathogens [217]. These problems led to the search for alternative approaches to control ETEC [217]. Anti-adhesive biomolecules that act as glycan receptor analogues are a promising alternative to antibiotics. Glycan receptors bind to glycolipids or glycoproteins on

the surface of host tissues and thus mediate adhesion of pathogens and toxins including the adherence of ETEC fimbriae to the epithelial cells [155,156]. Glycan receptor analogues bind to these glycan receptors and thus inhibit the initial stages of infection and bacterial colonization [155,156]. Anti-adhesive agents do not have bactericidal or bacteriostatic activity and therefore do not result in development of antimicrobial resistance [156,158].

Anti-adhesive agents that inhibit the adhesion of ETEC include human milk oligosaccharides (HMOs) as well as oligosaccharides in bovine colostrum which prevent ETEC adhesion in calves [218]. Major HMOs are composed of fucose, galactose, glucose, N-acetyl-glucosamine, and N-acetyl-neuraminic acid or sialic acid [219]. However, human milk is not commercially available and HMO analogs that are purified from bovine colostrum or produced with microbial cell factories are relatively expensive [219,220]. Alternative oligosaccharides or glycopeptides known to inhibit ETEC adhesion include glycans formed by *Limosilactobacillus reuteri,* glycopeptides obtained from ovomucin hydrolysis, and galactosylated chitosan oligosaccharides [216,146,74]. Ovomucin-derived glycopeptides prevent adhesion of porcine ETEC K88 fimbriae at minimum inhibitory concentration (MIC) of 2.5 g L^{-1} while β-galactosylated chitosan-oligosaccharides inhibit ETEC K88 adhesion at MIC of 0.22 g L^{-1} [74,216].

Bovine caseinomacropeptide (CMP) constitutes about 15-20 % of the total whey protein and represents the C-terminus of κ-casein obtained by specific hydrolysis of κ-casein with rennet. Glycosylated CMP (GMP) contains a high portion of sialic acid, which constitutes 7-9 % of its the total weight [221,127]. Bovine GMP also blocks the adhesion of diverse enteric pathogens to the intestinal mucosa including enterohemorrhagic *E. coli* (EHEC) O157 [147] and ETEC K88 adhesion in swine [75]. Bovine GMP prevents adhesion of porcine ETEC K88 fimbriae at MIC of 2.5 g L^{-1} [75].

In North Africa and in the Middle East, the production of camel milk cheese has increased; this increase in camel milk cheese production has also increased the amount of whey available as a by-product[21]. Comparable to other domestic animals, ETEC causes diarrhea in camel calves [222]. Camel milk is not as well studied as bovine milk and the chemical composition, and the biological activities of camel CMP are not described. Therefore, this study aimed to purify CMP from camel milk, to characterize its glycan composition, and to assess its activity in preventing adhesion of ETEC K88 adhesion to porcine erythrocytes.

5.2. Materials and methods

Purification of CMP

Bovine GMP was purchased from Davisco Foods International (Eden Prairie, MN, USA). CMP from Bactrian camels or dromedaries was purified from cheese whey that was prepared by treatment of reconstituted lyophilized milk from Bactrian camels (Inner Mongolia Agricultural University, China) or of reconstituted spray dried milk from dromedary camels (Al Ain Farms, Al Ain, UAE) with camel chymosin (Chr. Hansen, Bayswater, Australia), followed by separation of the whey using centrifugation (Figure 1). CMP was purified as previously described with some modifications [127], as illustrated in Figure 1. Briefly, about 25 mL whey with a pH of 6.2 was ultra-filtrated, dialyzed with 5 kDa membranes and then incubated in boiling water for 10-12 min to denature whey proteins. After cooling at room temperature (22 ˚C), precipitated protein was removed by centrifugation at 5, 000 ×g for 40 min at 20 ˚C. The pH of the supernatant was adjusted with 2M HCl to 4.3, the pI of camel milk casein, and precipitates were removed by centrifugation. The pH of the supernatant was adjusted to pH 3.0 with 2 M HCl and the solution was loaded on a 1.5 cm × 20 cm column of diethylaminoethyl (DEAE)-Sephadex A-25 (GE Healthcare, Chicago, IL). CMP was eluted using a linear gradient from 0 to 1 M NaCl. CMP eluting from the column

was detected by measuring the absorbance at 549 nm after derivatization with thiobarbituric acid [223]. Camel milk oligosaccharides were prepared with the same protocol from acidic whey that was not treated with chymosin.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out using 4-20 % gradient acrylamide ready to use gels (Mini-PROTEAN TGX Precast Protein Gels, Bio-Rad Laboratories, Hercules, CA, USA). CMP was dissolved in distilled water containing 2.5 % (v/v) mercaptoethanol to a concentration of 5 g L^{-1}, diluted 1: 4 (v/v) with SDS loading buffer, heated at 90°C for 5 min, and loaded on the gel. Proteins were separated for 50 min at 150 V and protein bands were stained with Coomassie Blue. Thermo Scientific PageRuler™ Prestained Protein Ladder (10-250 kDa), and Spectra™ Multicolor Low Range Protein Ladder (1.7- 40kDa) (Fisher Scientific) were used as molecular markers.

Reverse phase high performance liquid chromatography coupled to mass spectrometry (LC-MS).

LC-MS analyses of GMP were conducted by the Mass Spectrometry Laboratory of the Department of Chemistry at the University of Alberta. LC-MS was performed with an Agilent 1200 SL HPLC System and a Phenomenex Aeris 3.6 µm, WIDEPORE XB-C8, 200 Å, 2.1 x 50 mm guard column. The column was eluted at 0.5 ml min^{-1} and 40 °C with 0.1% (v/v) formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with the following linear gradient: 0 min, 5 % B; 0.5 min, 5% B; 5.5 min, 60 % B; 7 min, 98 % B, followed by washing for 2.8 min and re-equilibration. Mass spectra were acquired in positive mode of ionization using an Agilent 6220 Accurate-Mass TOF LC/MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source. Mass correction was performed for every individual spectrum using peaks at m/z 121.0509

and 922.0098 from the reference solution. Mass spectrometric conditions were drying gas 10 L min-1 at 325 °C, nebulizer 20 psi, mass range 100-3000 Da, acquisition rate of ~1.03 spectra/sec, fragmentor 225 V, skimmer 65 V, capillary 4000 V, instrument state 4 GHz High Resolution. Data analysis was performed using the Agilent Mass Hunter Qualitative Analysis software package version B.03.01 SP3.

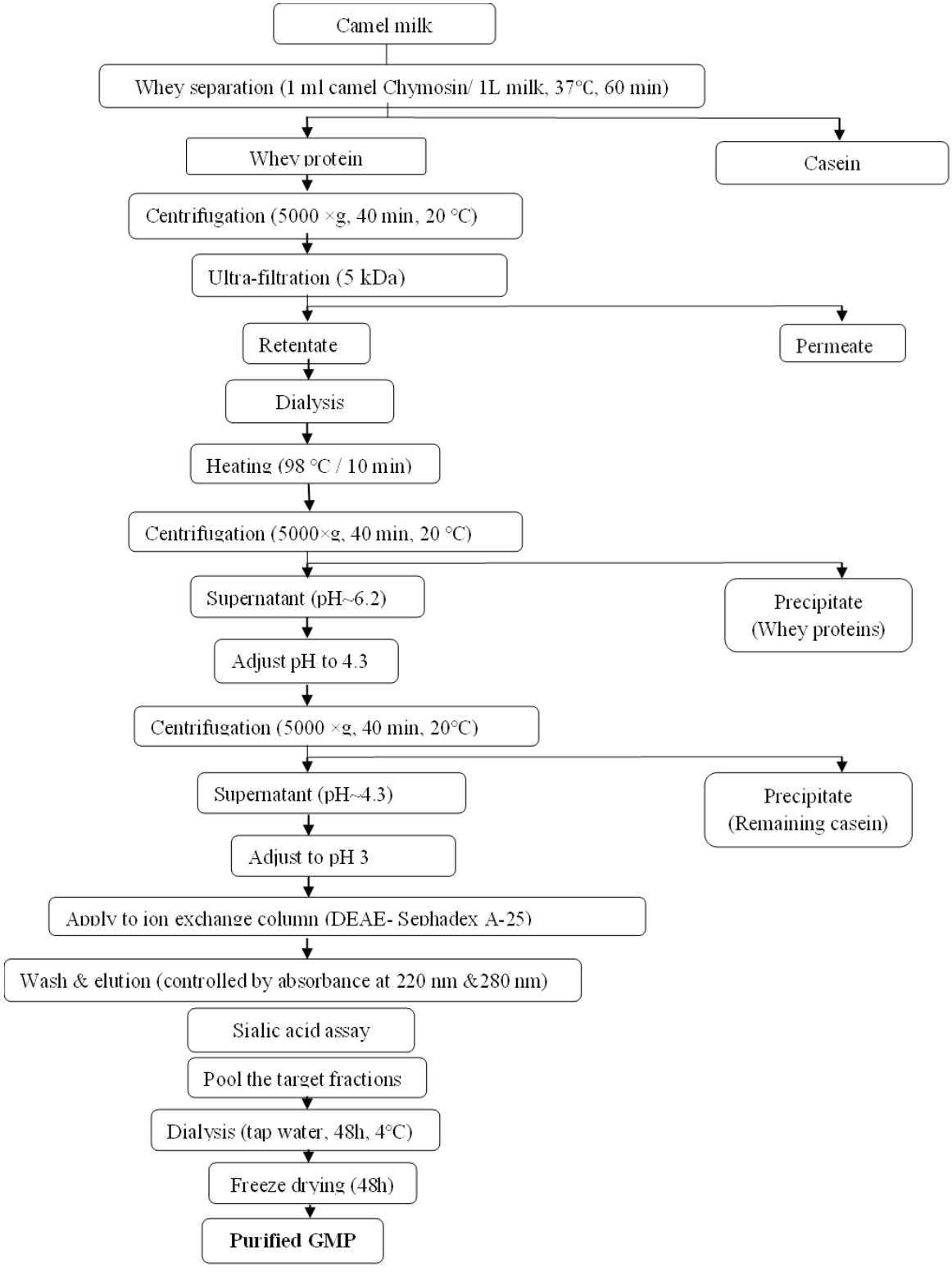

Figure 5.1: Flow diagram for the purification of the caseinomacropeptide (CMP) from camel milk

Determination of glycan composition.

To analyze monosaccharide content and distribution of GMPs from cows, Bactrian camels, and dromedaries, 20 µL of a 5 g/L solution were hydrolyzed by using 2 M trifluoroacetic acid (TFA). Briefly, samples were first dried with an evaporator (Eppendorf Concentrator, Hamburg, Germany) at 45 °C, followed by addition of 1 mL of 2 M TFA. and incubation 1 h at 121 °C. For the determination of N-acetylneuraminic acid, a reaction temperature of 70 °C was used for hydrolysis with 2 M TFA. After incubation, samples were evaporated and subsequently washed twice with 200 µL ethanol. The dried hydrolysates were finally dissolved in 200 µL of ultrapure water and analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS6000 system (Thermo Fisher Scientific, Waltham, MA.) equipped with a Dionex™ CarboPac™ PA20 column (150 mm x 3 mm i.d., 6.5 µm particle size, Thermo Fisher Scientific, Waltham, MA.). The eluents used for the gradient were A) ultrapure water, B) 10 mM sodium hydroxide, C) 200 mM sodium hydroxide and D) 200 mM sodium hydroxide with 200 mM sodium acetate. The flow rate was of 0.4 mL/min at 30 °C. Before every run, the column was rinsed with 100 % B for 10 min and then conditioned with 30 % A, and 70 % B for additional 10 min. After injection, samples were eluted with the following gradient: 0 - 27.5 min isocratic with 30 % A and 70 % B; 27.5-35 min linear to 100 % C; 35 - 45 min linear to 100 % D, 45 - 50 min isocratic 100 % D, 50 - 60 min isocratic with 100% C to remove the acetate from the column.

Bacterial strains and growth conditions.

A porcine ETEC expressing K88 fimbriae, *E. coli* ECL 13795, was used to determine the anti-adhesion activity. ETEC K88 was cultivated on Minca agar aerobically at 37 °C for 6 - 8 h. Cells were washed from the plates with 3 mL of phosphate buffered saline (PBS, 137 mM NaCl; 2.7

mM KCl; 10 mM Na2HPO4; 145 1.8 mM KH2PO4, pH 7.2). The cell density of the suspension was determined by measuring the optical density (OD) at 600 nm and adjusted to approximately 10^9 CFU mL^{-1} as described [74].

Hemagglutination assay to detect the impact of GMP on ETEC K88 adhesion to piglet erythrocytes.

Hemagglutination was performed in V-bottom 96-well polystyrene microtiter plates (Corning) as previously described [74]. Briefly, porcine whole blood cells (Innovative Research Inc., USA) were washed three times in PBS and erythrocytes were resuspended in PBS to a final density 5 % (v/v). ETEC K88 suspension (25 µL with about 109 CFU mL^{-1}) was added to the first column of the microtitre plates and diluted horizontally in ten two-fold serial dilutions. Samples or controls were dissolved at 10 g L^{-1} and diluted in 8 serial twofold dilutions in PBS. Different concentrations of the same sample or control (25 µL each) were added to the same column of the microtitre plate. The plates were incubated at room temperature (23 °C) for 5 min prior to addition of 25 µL of erythrocyte suspension. Plates were incubated overnight (16 h) at 4 °C before visual scoring of agglutination of erythrocytes as described [224]. Anti-adhesive activity was recorded if the sample or control solution increased the number of ETEC K88 cells that agglutinate erythrocytes at least four-fold. The lowest concentration of GMP with anti-adhesive activity was recorded as the minimum anti-adhesive concentration (MAC). Addition of PBS, bovine serum albumin (BSA), lactose, and oligosaccharides from acidic whey served as negative controls. Bovine GMP was used as a positive control.

Enzyme-Linked Immunosorbent Assay (ELISA) to test the ability of GMP to prevent ETEC K88 adhesion to porcine erythrocytes.

The ELISA assay was conducted in 96-well high binding microtiter plates (Corning) as previously described [74] with minor modifications to confirm the impact of GMP on ETEC K88 adhesion to piglet erythrocytes. Briefly, 100 µL of 5 % porcine erythrocytes were added to coat the high binding 96-well plate for 16 h, and plates were then blocked by addition of 200 µL of 3 % BSA, followed by incubation for 60 min at 4 ℃. GMP and controls were dissolved in PBS to 10 g L^{-1} and diluted in PBS in 8 serial twofold dilutions. ETEC suspensions were mixed with GMP or control solutions 1:1 (v/v) before 100 µL of the mixtures were added to the plate and incubated for 60 min at 4 ℃. Then 100 µL of 1:2000 diluted mouse anti *E. coli* K88A antibody (Bio-Rad Laboratories, Hercules, CA, USA) was added and incubated at 4 ℃ for 60 min. Then 100 µL of 1:1000 diluted goat antimouse IgG (H+L) secondary antibody (Invitrogen, Fisher Scientific, CA, USA) was added, followed by incubation for 60 min at 4 ℃. TMB substrate (50 µL) was then added to each well. The reaction was stopped after 30 min by adding 50 µL of 2M sulfuric acid, and the absorbance at 450 nm was determined with a Varioscan Flash Microplate reader (Thermo Scientific, CA, USA). Between each step of the above protocol, three washing steps with 200 µL of PBS were performed. Erythrocytes without ETEC suspension, erythrocytes with ETEC suspension but without samples, and ETEC suspension without erythrocytes were used as controls in addition to the same negative and positive controls that were also used in the hemagglutination assay.

Deglycosylation of GMP.

To remove the glycans (free oligosaccharides) from GMP, the *O*-glycosidase kit (P0733S, 40,000,000-units/mL, New England BioLabs, ON, Canada) was used with and without

neuraminidase (Sialidase) (11585886001, 5 U, Sigma, Mannheim, Germany). Briefly, 20 µg of GMP was mixed with 1 µL of 10X glycoprotein denaturing buffer in 10 µL H_2O. After denaturation of GMP denaturation at 100 °C for 10 min, 2 µL of 10X GlycoBuffer (2), 2 µL of 10 % NP40, 2 µL of Sialidase, and 3 µL of *O*-Glycosidase were added. The mixture was incubated for 3 hours at 37 °C. The enzyme kit that was used for protein deglycosylation hydrolyses O-glycosidic bonds of the disaccharide Gal-β-1-3GalNAc as well as larger oligosaccharides [225].

Statistical Analysis.

Bioassays were performed in triplicate biological repeats with three technical repeats, and the results are presented as means ± standard error. To determine the statistical differences between the samples and concentrations, p-values were calculated using Tukey Pairwise Comparisons at 95% Confidence in Minitab 19 (The differences between the conditions are considered significant if p-value < 0.05).

5.3. Results

CMP Purification.

CMP from Bactrian camels and dromedaries were purified with a protocol that was developed for bovine GMP and employs rennet- and heat-induced precipitation of casein and whey proteins, respectively, and ultra-filtration. Negatively charged CMP was then separated on an anion exchange column (Figure 5.2 A, B). CMP is glycosylated and phosphorylated (Figure 5.3). The predicted molecular weights (Mw) (https://peptidenexus.com/peptide) of non-glycosylated CMPs from Bactrian camel and cows were 6.774 kDa and 6.707 kDa, respectively. The mass spectra obtained by ESI-LC-MS included the predicted ion species of non-glycosylated CMP for Bactrian camels and cows at 6.777 kDa and 6.787 kDa respectively; additional peaks were observed that

likely represent the peptides with different levels of glycosylation and phosphorylation (Figure S1; Appendices). The purity of purified Bactrian camel GMP, dromedary GMP, and bovine GMP were assessed by SDS-PAGE (Figure 5.4).

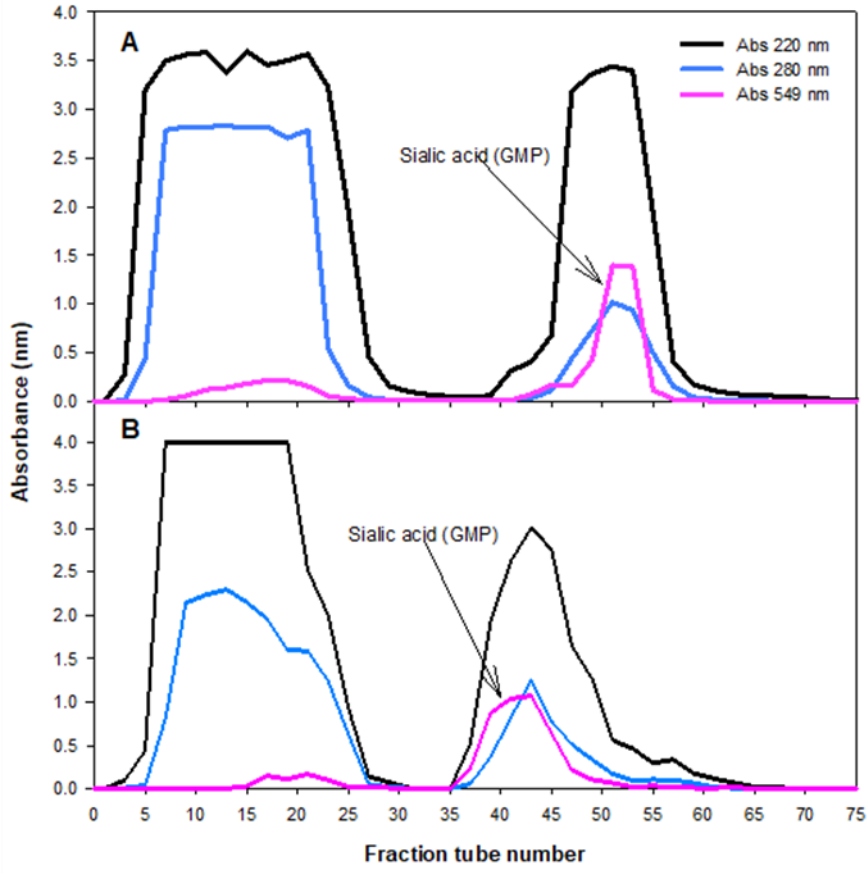

Figure 5.2: Separation of the GMP from milk of Bactrian camels (**Panel A)** and dromedaries (**Panel B)** on a diethylaminoethyl (DEAE)- Sephadex A-25 column. Sialylated oligosaccharides were eluted with 0.5 M NaCl and detected at 549 nm after derivatization. Camel milk oligosaccharides were prepared as negative control from milk that was not treated with rennet.

The pattern of all GMPs samples presented visible regular thin band located at about ~14 kDa, corresponding to the dimeric GMP form composed of 2 GMP monomers. The separation of bovine GMP and GMP from dromedary milk produced additional bands with an apparent Mw of 14 – 30 kDa.

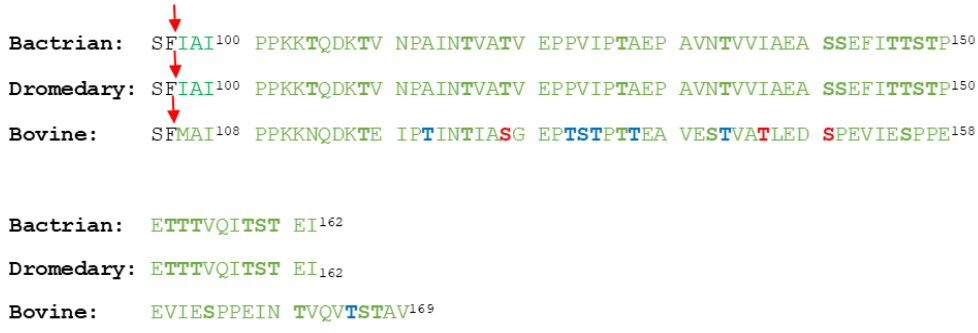

Figure 5.3: Amino acid sequence of the caseinomacropeptide (C-terminus of κ-casein) from Bactrian camel (L0P304), dromedary (P79139), and cows (P02668). Green colored amino acids indicate the caseinomacropeptide (CMP); bold residues indicate potential sites for glycosylation or phosphorylation. Bold blue-colored letters correspond to amino acids that were shown to be glycosylated in variant A of bovine GMP; red-colored letters correspond to amino acids that were shown to be phosphorylated in variant A of bovine GMP.[136] The red arrow indicates the cleave sites of chymosin.

Composition of the glycans in GMP from cattle, Bactrian camels, and dromedaries.

To determine the glycan composition of the GMPs, glycans were hydrolyzed with 2 M TFA, and the concentration of resulted monosaccharides was determined (Table 5.1). Hydrolysis of glycans with 2 M TFA not only hydrolyses the glycosidic bonds but also partially or completely deacetylates N-acetylglucosamine and N-acetylgalactosamine; these are therefore detected as the corresponding amino sugars. The amount of total monosaccharides from bovine GMP was less

than 50 % of the amount of monosaccharides from Bactrian camel and dromedary GMPs. Fucose and glucosamine were detected in GMP from Bactrian camels and dromedaries but absent (fucose) or only present in a low concentration (glucosamine) in bovine GMP. Galacturonic acid and glucuronic acid were detected in GMP from Bactrian camels only (Table 5.1).

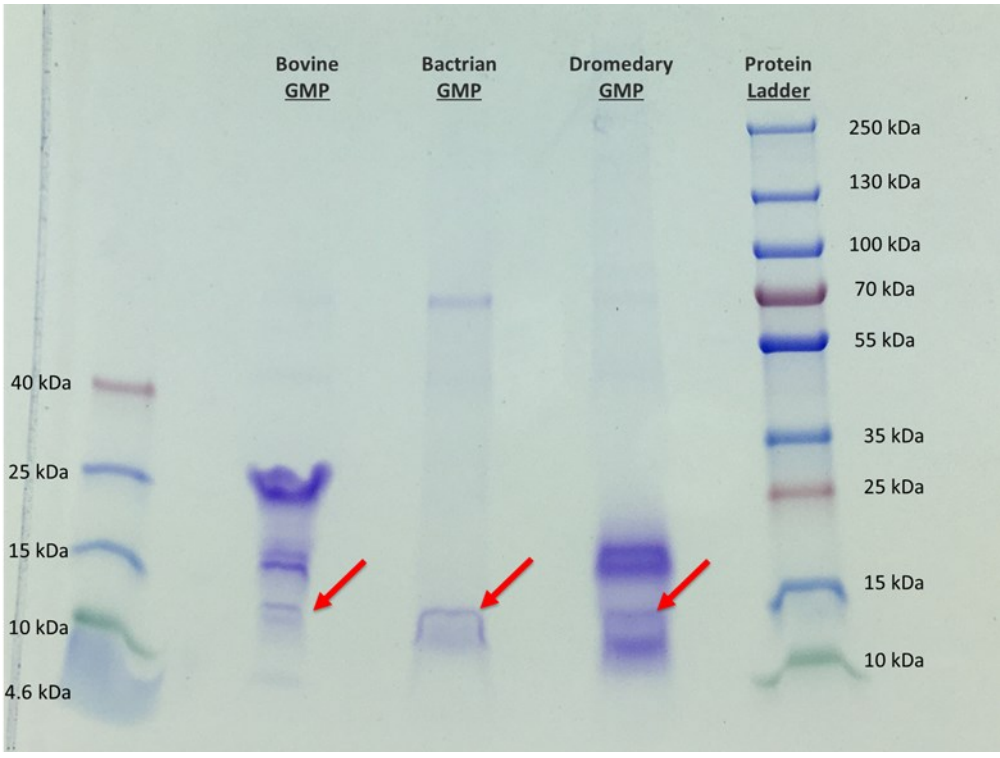

Figure 5.4 Separation of purified GMP from Bactrian camel and dromedary, and of commercial bovine GMP by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); red arrows indicate the pure GMP.

The monosaccharide composition after hydrolysis of GMP from Bactrian camels and dromedaries also differed qualitatively and quantitatively, e.g., GMP from dromedaries contained more glucose than GMP from Bactrian camels.

Table 5.1 Composition of monosaccharides after hydrolysis of bovine, Bactrian camel, or dromedary GMP with 2 M TFA. The error was calculated from the Residual Standard Error of the linear or linear quadratic regression of the standard curves.

amount (mg sugar / g GMP)	Bovine	Bactrian camel	Dromedary
Gal	22.13 ± 0.35	58.63 ± 0.76	53.15 ± 0.23
GalN	20.54 ± 0.18	38.53 ± 0.17	33.23 ± 0.23
NeuNAc[a)]	39.06 ± 0.27	52.98 ± 0.25	64.68 ± 0.28
GlcN	1.24 ± 0.16	19.75 ± 0.22	15.10 ± 0.05
Glc	1.48 ± 0.35	6.81 ± 0.31	12.03 ± 0.37
GalA	n.d[b)]	0.73 ± 0.16	n.d.
Fuc	n.d.	0.43 ± 0.10	0.60 ± 0.05
GlcA	n.d.	traces[c)]	n.d.
GlcNAc, GalNAc and Neu5GC	n.d.	n.d.	n.d.
Sum of all sugars	84.4	178.0	178.2

[a)] The NeuNAc concentration was determined after hydrolysis at 70 °C

[b)] n.d., not detected

[c)] Concentration between limit of detection and limit of quantitation.

Impact of GMP on ETEC K88 adhesion to porcine erythrocytes.

The hemagglutination assay was performed for GMP from Bactrian camel and dromedary milk, using bovine GMP, oligosaccharides from camel acidic whey, bovine serum albumin (BSA) and lactose served as controls. The strongest anti-adhesive activities against ETEC K88 were observed for Bactrian camel GMP (Table 5.2). BSA and lactose had no anti-adhesive activities against ETEC K88. However, oligosaccharides from Bactrian camel acidic whey that was prepared without chymosin treatment had a lower minimum anti-adhesive concentration than the positive control (Table 5.2).

Table 5.2 Inhibition of erythrocyte agglutination by ETEC K88 strain with Bactrian camel GMP, dromedary camel GMP, bovine GMP, oligosaccharides from camel acidic whey, bovine serum albumin (BSA), and lactose. Data for bovine and camel GMPs and acidic oligosaccharides are shown as means ± standard deviation of three independent preparations from dry milk. Values obtained with different compounds or preparations at the same concentration differ significantly ($P<0.05$) if they do not share common letter

sample	**minimum concentration for erythrocyte agglutination with ETEC (g/L)**
Bactrian camel GMP[A]	$0.24^{b} \pm 0.02$
Dromedary camel GMP	$0.28^{b} \pm 0.03$
Bovine GMP	$5.52^{a} \pm 1.06$
Oligosaccharides from milk of Bactrian camel	$0.87^{b} \pm 0.15$
Lactose	> 10
BSA	> 10

[A] The preparations of camel GMP included about 10 % acidic oligosaccharides

An ELISA was used to confirm the activity of GMP from milk of Bactrian camels and controls in preventing ETEC K88 adhesion to porcine erythrocytes. Comparable to the hemagglutination assay, the highest anti-adhesive activity was observed for Bactrian GMP, followed by oligosaccharides from Bactrian camel acidic whey, bovine GMP, lactose, and BSA respectively (Figure 5.5). At a concentration of 10 g L^{-1}, Bactrian camel GMP reduced ETEC adhesion by about 75 %: at 0.125 g L^{-1}, Bactrian camel GMP still significantly reduced ETEC adhesion (Figure 5.5). The results from both the hemagglutination and ELISA assays demonstrated that GMP from Bactrian camels and dromedaries as well as oligosaccharides from Bactrian camel acidic whey had higher anti-adhesive activities when compared to bovine GMP.

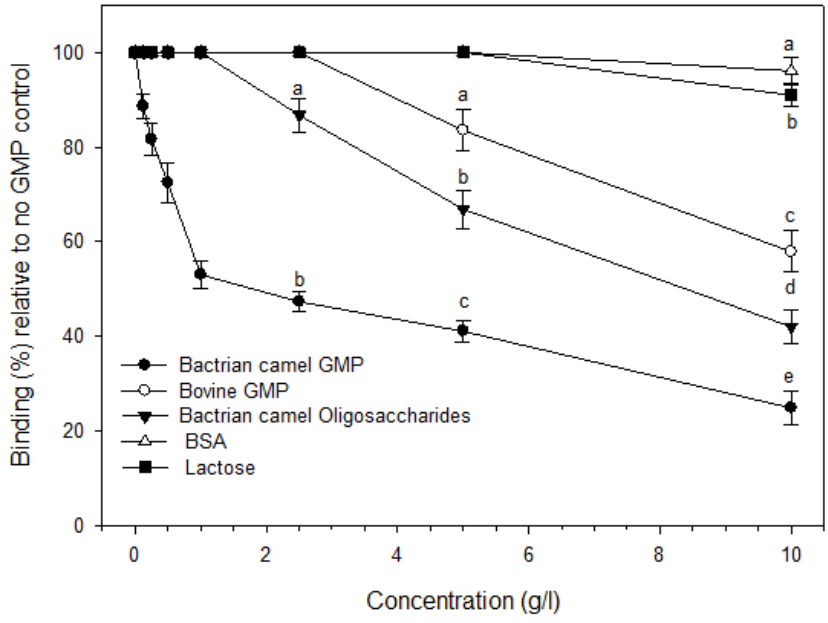

Figure 5.5: Quantification of E. coli K88 ECL13795 binding to porcine erythrocytes with ELISA targeting K88 antibodies. ETEC were incubated with erythrocytes without addition of GMP (no GMP control) or with addition of different concentrations of GMPs. Lactose, bovine serum albumin, and acidic oligosaccharides from milk from Bactrian camels served as controls. Based on the yield of GMP and acidic oligosaccharides from the milk of Bactrian camels, 50 and 5 mg / L, respectively, the GMP preparation from Bactrian camels included about 10% of acidic oligosaccharides in addition to the GMP. Values obtained with different compounds or preparations at the same concentration differ significantly ($P<0.05$) if they do not share a common letter. Results are reported as means ± standard deviation of three independent assays.

Deglycosylation of GMP.

To determine whether the effect on ETEC adhesion requires that oligosaccharides are bound to the caseinomacropeptide (peptide backbone), the activity of GMPs was compared to the activity remaining after enzymatic deglycosylation with sialidase and O-glycosidase. The resulting free

sugars and deglycosylated GMPs (caseinomacropeptide) were redissolved in PBS at 10 g L^{-1} and diluted to determine their biological activities (Table 5.3). The activity of the glycosylated GMPs was consistent with results shown in Table 5.2; glycan hydrolysis with sialidase and O-glycosidase (Table 5.3) or with O-glycosidase only (data not shown) eliminated anti-adhesive activity.

Table 5.3 Inhibition of erythrocyte agglutination by ETEC K88 strain with Bactrian camel GMP, dromedary camel GMP, and bovine GMP before and after enzymatic deglycosylation. Data for camel GMPs and bovine GMP are shown as means ± standard deviation of three independent assays for the same preparations. Values obtained with different compounds or preparations differ significantly ($P<0.05$) if they do not share a common superscript.

sample	**minimum concentration for erythrocyte agglutination with ETEC (g/L)**	
	Before deglycosylation	**After deglycosylation**
Bactrian camel GMP	0.25^{b}± 0.00	> 10
Dromedary camel GMP	0.25^{b}± 0.00	> 10
Bovine GMP	5.00^{a} ± 0.00	> 10
Control (just enzyme/ no GMP)	> 10	> 10

5.4. Discussion

Camel is an important source of milk in many countries including some of the developing countries where childhood diarrhea caused by ETEC is very common [42,226]. Whey is a byproduct of camel milk cheese production [42] and is thus an inexpensive source of bioactive compounds including GMP. In the present study, purification of camel and dromedary GMP was achieved with a protocol that was developed for purification of bovine GMP [127]. The experimental Mw of non-glycosylated bovine CMP that was observed by LC-MS/MS, 6.787 kDa, matches prior observations [227,228], LC-MS data for the GMP from Bactrian camels is not available.

The average molecular weight (Mw) of glycosylated bovine GMP is 7.5 kDa [229]. The SDS-PAGE analysis indicates that the CMP from Bactrian camels and dromedaries form dimers as was previously shown for bovine and goat CMP as a result of self-assembly [230,135,128]. The additional bands present in dromedary CMP and bovine CMP correspond to trimeric and tetrameric CMP. The observation of multimeric aggregated CMP was reported previously after separation of bovine CMP, which migrates on SDS-PAGE as a mixture of polymers [230,231,232]. It was suggested that hydrophobic interactions stabilize the CMP dimers while electrostatic bonds additionally stabilize the multimeric aggregates of CMP [230,231,232].

The multimeric aggregates of CMP were least abundant in the sample prepared from milk of Bactrian camels; this was also the only sample that was not pasteurized prior to preparation of the CMP, indicating that pasteurization contributes to the aggregation of bovine and camel CMP. The monosaccharide composition of bovine GMP matches prior reports on its glycan composition [142,141]. Bovine GMP is glycosylated with a disaccharide composed of galactose and N-acetylgalactosamine (GalNAc), which is decorated with one or two N-acetylneuraminic acid (NeuNAc) moieties. Glycosylation with oligosaccharides that additionally include fucose and N-acetylglucosamine (GlcNAc) was reported in GMP from bovine colostrum[133]. GalNAc and GlcNAc were detected as the deacetylated amino sugars galactosamine (GalN) and glucosamine (GlcN). This is the result of extensive deacetylation during TFA hydrolysis [233,234,235]. The confounding in the results by naturally occurring GalN and GlcN is unlikely because they are highly reactive in the Maillard reaction [236] and were not identified in glycoproteins in milk [133]. NeuNAc is also degraded when high temperatures are applied during acid treatment [237].

Therefore, the reaction temperature during TFA hydrolysis was reduced to 70 °C which allowed the quantification of NeuNAc in the different GMPs. The monosaccharide content of GMPs from

Bactrian camels and dromedaries was about twice as high as bovine GMP, corresponding to the higher number of potential glycosylation sites in these two species (Fig. 5.3). The difference is more significant for galactose, GalNAc, NeuNAc and GlcNAc. For instance, the content of GlcN in Bactrian camel and dromedary is 6 - 8 fold higher than in bovine GMP (Table 5.1). Many of the biological activities of GMP are mediated by the glycan structure [133], therefore, the increased glycan content of GMP from *Camelus* species may also impact their biological activity.

ETEC K88 infect young piglets and calves [157], and K88 fimbriae mediate the binding of *E. coli* ECL13795 to glycan receptors [152]. Porcine aminopeptidase N is a receptor for F4 (K88) fimbriae; in addition, surface glycan oligosaccharides composed of GalNAc, GlcNAc, GalN, and N-acetylmannosamine were proposed as receptors for ETEC K88 adhesion [152,157]. GMP from both Bactrian camels and dromedaries showed potent anti-adhesive activity at concentrations of about 0.25 g L^{-1}, which is about 20-fold lower than the effective concentration of bovine GMP [75,215]. This increased activity *in vitro* likely relates to the increased glycosylation and / or differences in the glycan composition and may also translate to an increased activity *in vivo*. Bovine GMP also reduced the attachment of ETEC K88 *in vivo* [75,238] and improved growth performance of *E. coli* K88-challenged piglets [75,238].

This relationship of glycan structure to biological activity was confirmed by comparison of the activity of glycosylated GMPs to the activity of free oligosaccharides and GMPs after enzymatic deglycosylation (caseinomacropeptide) (Table 5.3). Free oligosaccharides from camel milk were less active than GMP and deglycosylation of GMP strongly reduced the prevention of ETEC adhesion. The higher activity of the more densely glycosylated camel GMP, and the strong decrease of activity after deglycosylation suggests that not only the structure but also the density spacing of glycans on the peptide backbone are important for anti-adhesive activity. The

topological spacing of glycans is recognized as an important factor affecting the anti-adhesive activity of glycopeptides [138,147,137,139].

5.5. Conclusion

Hemagglutination and ELISA assays indicated that the anti-adhesive activity of GMP from Bactrian camels and dromedaries was substantially higher than the activity of bovine GMP. Free oligosaccharides from camel milk that were prepared as a control were also active but at a higher concentration when compared to GMP. Deglycosylation of GMP suggested that the spatial arrangement of glycans on the peptide backbone contributes to anti-adhesive activity. The *in vitro* anti-adhesive activity of bovine GMP was confirmed to also reduce ETC K88 adhesion *in vivo* [75]. Therefore, it is likely that the *in vitro* activity of GMP from *Camelus* species (this study) also translates to *in vivo* activity in swine. ETEC that infect humans, however, use different fimbriae with different binding specificity when compared to porcine ETEC [154,136], therefore, the use of CMP and GMP from camels and dromedaries remains subject to future investigations.

Chapter 6. **General Discussion and Conclusion**

Milk-whey is an abundant by-product of the cheese and casein production process. Cheese making is found in ancient Greek mythology and evidence of cheese making was also found in other ancient works dating back more than 7,000 years [239]. Whey represents around 85–90% of milk volume because the primary content of whey is water, and hundreds of millions of tons of whey accumulate as a major by-product of the casein and cheese industries[240]. Volumes of milk-whey are growing (>2% per year) at around the same rate as volumes of milk worldwide[240]. Whey has been considered a waste product in developing countries and requires expensive treatment before its disposal into the environment. In some cases, milk-whey is sprayed onto fields, discharged into the ocean, sea, or rivers, discharged into sewage systems, and processed into relatively low-value commodities such as whey powder or whey protein concentrates for use as a food ingredient in animal feed. Dairy industries, therefore, are looking for an economical management approach to find an alternative use of this resource.

The environmental regulations in western countries and some developing countries prohibited disposal of untreated milk-whey. These regulations forced the dairy companies to think of how they can make better use of this enormous volume of whey. During the last 60 years, research has focused on the whey proteins and the conversion of lactose. In parallel, there are many factors helping to increase the value of milk-whey and transformation of milk-whey from a nuisance into valuable bio-functional products. Science knowledge, sophistication, technology advances, higher consumer demands, revolution in functional foods, and food marketplace expansion have created opportunities for whey. This transformation of milk-whey from nuisance into a valuable bio-functional source was done in two stages with the first approach to use the whey and the second then to explore the beneficial function of incorporating it into value added products.

In the last decades, the prevalence of food-related chronic diseases including cardiovascular disease, alcoholic fatty liver disease, cancer, obesity, diabetes mellitus, and hypertension has increased worldwide [52,68]. Thus, the global market size and consumer demands for functional food has increased dramatically. In this growing marketplace, the food companies are demanding economical, novel, high-quality, and scientifically substantiated ingredients. Studies have suggested that bioactive peptides have a favorable effect on the functions of various organs and offers multiple biological and physiological benefits with a wide range of biological activities[69]. Bovine whey holds approximately 55% of the nutrients of milk and contains approximately 100% of lactose and 20% of the original milk protein and therefore represents a significant lactose and protein source[240]. As a result, whey is an inexpensive source of high-nutritional quality protein and bioactive peptides for the food and health industries [79]. Sweet whey contains ~5% caseinomacropeptide (CMP), which is the important bioactive peptide with many health benefits like anti-adhesion activity against ETEC[74,75]. GMP is used as source of sialic acid, however, as sialic acid constitutes 7% - 9% of the total GMP [133].

Milk-whey is recognized as a dietary source for health beneficial bioactive compounds. Whey contains a rich mixture of soluble proteins with many chemical, physical and functional properties. Whey proteins has a special biological value because of high content of essential amino acids, amino acids containing sulphur, and hydrophobic amino acids. Numerous investigation of many whey proteins and their derived peptides revealed potential health benefits [161]. Despite the large number of studies on milk-derived bioactive peptides, most of these studies focus on bovine milk only[21]. A handful of studies have investigated the bioactive peptides from sheep, goats, horse, or camel milk [21], reflecting that milk from these animals make only relatively small contribution to the overall production of liquid milk or fermented dairy products [80].

Camel milk attracted more consideration due to its therapeutic properties [42,44]. Most of the research on camel milk has focused only on casein and raw milk, however, only few studies focus on whey proteins from camel milk [21,4]. The production of camel milk fermented products and cheese has dramatically increased in, Asia, Africa, and middle east countries in addition to Turkish and Australia [42,46]. However, the full potential of the whey protein resulted from camel milk cheese has not yet been explored [47], and only a little credit will be given to the whey protein when used as animal feed [241].

In addition to Bactrian camel and dromedary there are two other tame types of small camelids from Camelidae family, Llama and Alpaca. These camels are called the "South American Camelidae" or "New World camels" [1,3].

This thesis investigated the enzymatic hydrolysis and successive chromatographic fractionation of camel milk whey protein to produce bioactive peptides and glycopeptides and determined the biological activities of these produced peptides. The antioxidant activity, antihyperglycemic activity, and anti-adhesion activity against bacteria of these peptides from the camel milk hydrolysates were evaluated. A literature review was performed and documented that the camel milk whey proteins have a high content of hydrophobic amino acids and have a balance between essential and non-essential amino acids and contain most of the essential amino acids (i.e., Phe, Val, Thr, Try, Met, leu, Ile, Lys, and His) with high concentration of Phe, Val, Leu, and Lys. On the other hand, camel milk whey proteins are rich in Glu and Pro as a non-essential amino acid. These given characteristics and facts supported the identified activities and led us to conclude that the camel milk whey protein is a promising candidate to produce favourable bioactive peptides and that it can become a major part of a functional food as an antioxidant agent, starch digestion inhibitor, and anti-adhesion agent [3,242].

In conclusion, although camel milk whey protein is a by-product of dairy industry, it could be an inexpensive source of high-nutritional quality protein and bioactive peptides for the food and health industries. Preliminary assessment of the antioxidant properties of camel milk whey protein hydrolysates indicates that they are not fundamentally different from those derived from bovine milk. Flavourzyme hydrolysates can be digested effectively by pepsin to get peptides with high antioxidant activity. The antioxidant activities of peptides were negatively affected by trypsin digestion. This study also revealed that the molecular weight is the most likely determining factor for antioxidant activities of peptides. Camel milk whey protein hydrolysates have a potent inhibitory effect on starch digestion. Separation of the positively charged peptide with hydrophobic amino acids identified two peptides, LALDIEIATYR and VLDELTLAR, that inhibited starch digestion more strongly than the unfractionated whey protein hydrolysate.

The monosaccharide content of glycomacropeptides (GMP) from Bactrian camels and dromedaries was about twice as high as that of bovine GMP. Glycans from camels like fucose and N-acetylglucosamine were absent in bovine GMP. GMP from both camel species prevented the childhood and piglets' diarrhoea (Enterotoxigenic *Escherichia coli*) through anti-adhesion. Camel milk GMP inhibited the adhesion of ETEC to porcine blood cells at a concentration that is about 20-fold lower than that of bovine GMP. This increased activity likely relates to the increased glycosylation and the glycan spacing, and/or to differences in the glycan compositions.

All together, this research proved the hypothesis, and confirmed *in vitro* the efficacy of camel milk whey protein-derived bioactive peptides against oxidative stress as an antioxidant agent, starch digestion as a starch digestion inhibitor, and adhesion activity against enteric pathogens as an antiadhesive agent. This research improves the understanding, how glycomacropeptide prevents the adhesion of pathogenic bacteria. This research improved the knowledge about the mechanism

of starch digestion inhibition and determined the responsible amino acid sequences. To the best of our knowledge, there are no peptides sequences determined in clinical studies confirming the potential health properties of camel milk.

There are many limitations of this research. The main chapters of this research were done during Covid-19 closure time led to limit this research on *in vitro* tests. Not availability of fresh camel milk in north America and the camel milk samples used in this research were freeze and spray dried, and the Llama and Alpaca milk are not available in commercially and several unsuccessful attempts to get milk samples from Llama and Alpaca farms were done. In literature, the experimental designs that used in the researches on camel milk do not allow conclusions as to whether the observed effects are attributable to protein and bioactive peptides or not, and that made the comparison and references are likely limited on bovine milk.

Therefore, to explore the potential bioactive peptides from camel milk designed *in vivo* studies on camel milk-derived protein hydrolysates are required. Further, more randomized clinical trials are required to realize the full potential activity of bioactive peptides derived from milk, and to determine the bioavailability of ingested bioactive peptides.

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Appendices

Table S1: Potential antioxidant peptides derived from caseins and whey proteins of camel, cow, goat, and sheep milk predicated by BIOPEP database (http://www.uwn.edu.pl/biochemia/index.php/pl/Biopep)

Protein fraction	Amino acid sequences of antioxidant peptides			
	Cow milk	Camel milk	Goat milk	Sheep milk
Milk protein:				
Casein:				
α -casein	ID: P02662 120 LH 121 143AY 144 158 AY 159 144YFYPEL 149 39 EL 40 141 EL 142 148EL149 164 WY 165 165 YYV 167 164 WYY 166 144 YFY 146 120 LHS 122 42 KD 43 147 PEL 149 146 YPEL 149	ID: O97943 100LH101 160HL161 157AY158 134 LY135 189AH190 30EL31 46EL47 99EL100 178WY179 178WYY180 100LHR102 123VKL125 149VKV151 48KD49 84KD85	ID: P18626 91 YLGY 94 120 LH 121 143 AY 144 158 AY 159 129 AH 130 39 EL 40 141 EL 142 164 WY 165 165 YYL 167 164 WYY 166 144 YFY 146 120 LHS 122 42 KD 43 101 LK 102 144 YFYPQL 149	ID: P04653 120 LH 121 143 AY 144 158 AY 159 129 AH 130 39 EL 40 141 EL 142 164 WY 165 165 YYL 167 164 WYY 166 144 YFY 146 120 LHS 122 42 KD 43 101 LK 102 144 YFYPQL 149 163 AW 164

	^{145}FYPEL^{145} ^{101}LK^{102} ^{163}AW^{164} ^{198}LW^{199} ^{170}GTQY^{173} ^{158}AYPS^{161} ^{154}YQLD^{157} ^{93}GYLEQ^{97} ^{146}YPELF^{150} ^{100}LKKY^{104} ^{20}LLR^{22} ^{98}LLR^{100} ^{154}YQL^{156} ^{153}FYQL^{156}	^{114}IR^{115} ^{47}LK^{48} ^{87}LK^{88} ^{177}AW^{178}	^{163}AW^{164} ^{198}LW^{199} ^{170}GTQY^{173} ^{158}AYPS^{161} ^{154}YQLD^{161} ^{93}GYLEQ^{97} ^{100}RLKKY^{104} ^{20}LLR^{22} ^{98}LLR^{100} ^{154}YQL^{156} ^{153}FYQL^{156} ^{17}NEN^{19} ^{102}KKY^{104}	^{198}LW^{199} ^{170}GTQY^{173} ^{158}AYPS^{161} ^{154}YQLD^{161} ^{93}GYLEQ^{97} ^{100}RLKKY^{104} ^{20}LLR^{22} ^{98}LLR^{100} ^{154}YQL^{156} ^{153}FYQL^{156} ^{17}NEN^{19} ^{102}KKY^{104}
B-casein	ID: P02666 ^{133}LH^{134} ^{134}HL^{135} ^{98}VSKVKEAM^{105} ^{192}LY^{193} ^{177}AVPYPQR^{183} ^{2}EL^{3} ^{5}EL^{6} ^{44}LQ^{45} ^{179}PYPQ^{182} ^{133}LHL^{135} ^{147}PHQ^{149} ^{59}VY^{60} ^{178}VPYPQ^{182} ^{199}PVRGPFPIIV^{208} ^{183}RDMPIQA^{188}	ID: Q9TVD0 ^{134}LH^{135} ^{205}LH^{206} ^{135}HL^{136} ^{50}IY^{51} ^{180}PYPQ^{183} ^{134}LHL^{136} ^{145}MY^{146} ^{60}VY^{61} ^{179}VPYPQ^{183}	ID: P33048 ^{133}LH^{134} ^{134}HL^{135} ^{190}LY^{191} ^{169}KVLPVPQK^{176} ^{170}VLPVPQK^{176} ^{191}YQEP^{194} ^{191}YQEPVLGP^{194} ^{5}EL^{6} ^{44}EL^{45} ^{133}LHL^{135} ^{59}VY^{60} ^{181}RDMPIQ^{186}	ID: P11839 ^{133}LH^{134} ^{134}HL^{135} ^{190}LY^{191} ^{169}KVLPVPQK^{176} ^{170}VLPVPQK^{176} ^{191}YQEP^{194} ^{191}YQEPVLGP^{194} ^{5}EL^{6} ^{44}EL^{45} ^{133}LHL^{135} ^{59}VY^{60} ^{181}RDMPIQ^{186}
K-casein	ID: P02668 ^{121}HPHL^{124} ^{80}PYY^{82}	ID: P79139 ^{69}LH^{70} ^{42}YYQ^{44}	ID: P02670 ^{100}HPHL^{103} ^{59}PYY^{61}	ID: P02669 ^{100}HPHL^{103} ^{59}PYY^{61}

	^{119}HPH^{121} ^{121}HPH^{123} ^{123}HL ^{124117}ARHPHPHLSFM^{127} ^{81}YYA^{83} ^{63}YYQ^{65} ^{62}NYY^{64} ^{122}PHL^{124} ^{34}KD^{35} ^{49}IQY^{51} ^{51}YVL^{53} ^{30}IR^{31} ^{67}KP^{68} ^{84}KP^{85} ^{186}TSTA^{189} ^{47}IPIQYVL^{53} ^{82}YAKPA^{86} ^{117}ARHPHP^{122} ^{118}RHPIP^{122} ^{55}RYPS^{58} ^{54}SRYPS^{58} ^{82}YAKP^{85} ^{52}VLSRYPS^{58} ^{145}TIASGEP^{151} ^{59}YGLN^{62}	^{41}NYY^{43} ^{69}LHA^{71} ^{67}IR^{68} ^{63}KP^{64} ^{34}RYPS^{37} ^{33}SRYPS^{37} ^{61}YAKP^{64}	^{98}HPH^{100} ^{100}HPH^{102} ^{102}HL^{103} ^{96}ARHPHPHLSFM^{106} ^{60}YYA^{62} ^{42}YYQ^{44} ^{41}NYY^{43} ^{101}PHL^{103} ^{13}KD^{14} ^{112}KD^{113} ^{28}IQY^{30} ^{30}YVL^{32} ^{63}KP^{64} ^{26}IPIQYVL^{32} ^{96}ARHPHP^{101} ^{97}RHPHP^{101} ^{34}RYPS^{37} ^{33}SRYPS^{37} ^{61}YAKP^{64} ^{31}VLSRYPS^{37} ^{38}YGLN^{41}	^{98}HPH^{100} ^{100}HPH^{102} ^{102}HL^{103} ^{96}ARHPHPHLSFM^{106} ^{60}YYA^{62} ^{42}YYQ^{44} ^{41}NYY^{43} ^{101}PHL^{103} ^{13}KD^{14} ^{112}KD^{113} ^{28}IQY^{30} ^{30}YVL^{32} ^{63}KP^{64} ^{26}IPIQYVL^{32} ^{96}ARHPHP^{101} ^{97}RHPHP^{101} ^{34}RYPS^{37} ^{33}SRYPS^{37} ^{61}YAKP^{64} ^{31}VLSRYPS^{37} ^{38}YGLN^{41}
GMP	^{60}TSTA^{63} ^{19}TIASGEP^{25}	----	^{7}KD^{8}	^{7}KD^{8}
Whey protein				
A-lactalbumin	ID: P00711 ^{125}AHK^{127} ^{125}AH^{126} ^{30}EL^{31}	ID: P00710 ^{106}AHK^{108} ^{106}AH^{107} ^{11}EL^{12}	ID: P00712 ^{106}AHK^{108} ^{106}AH^{107} ^{67}PHS^{69}	ID: P09462 ^{106}AHK^{108} ^{106}AH^{107} ^{11}EL^{12}

	86 PHS 88 32 KD 33 81 KD 82 31 LK 32 34 LK 35	13 KD 14 12 LK 13 108 KP 109 64 NEN 66	13 KD 14 16 KD 17 62 KD 63 12 LK 13 15 LK 16	67 PHS 69 13 KD 14 16 KD 17 62 KD 63 12 LK 13 15 LK 16
β-lactoglobulin	ID: P02754 61 EL 62 35 WYSLAMAASDI 45 161 MHIRL 165 58 YVEEL 62 35 WY 36 35 WYS 37 35 WYSL 38 35 WYSLA 39 35 WYSLAM 40 35 WYSLAMA 41 163 IR 164 62 LKP 64 62 LK 63 156 LK 157 63 KP 64 57 VY 58 113 TDY 115 34 TW 35	--------	ID: P02756 19WYSLAMAASDI29 45 EL 46 145 MHIRL 149 42 YVEEL 46 19 WY 20 19 WYS 21 19 WYSL 22 19 WYSLA 23 19 WYSLAM 24 19 WYSLAMA 25 147 IR 148 46 LKP 48 46 LK 47 140 LK 141 47 KP 48 41 VY 42 97 TDY 99 18 TW 19 17 GTW 19 59 QKW 61 61 WEN 63 105 FC 106 64 GEC 66 65 ECA 67 66 CAQ 68 102 YLL 104	ID: P67976 45 EL 46 145 MHIRL 146 42 YVEEL 46 147 IR 148 46 LKP 48 46 LK 47 140 LK 141 47 KP 48 41 VY 42 97 TDY 99 18 TW 19 17 GTW 19 59 QKW 61 61 WEN 63 105 FC 106 64 GEC 66 65 ECA 67 66 CAQ 68 102 YLL 104 104 LFC 106 105 FCM 107 106 CME 108 117 LAC 119 118 ACQ 120 119 CQC 121 120 QCL 122

			104 LFC 106 105 FCM 107 106 CME 108 117 LAC 119 118 ACQ 120 119 CQC 121 120 QCL 122 121 CLV 123 143 LPM 145 41 VYV 43 42 YVE 44 98 NENDYK 100 100 KKY 102 101 KYL 103 145 MHI 147 15 VAGTWY 20	121 CLV 123 143 LPM 145 41 VYV 43 42 YVE 44 88 NEN 90 98 DYK 100 100 KKY 102 101 KYL 103 145 MHI 147
Lactoferrin	ID: D0VAV0 612 LH 613 246 HL 247 588 HL 589 165 AY166 318 LY 319 81 IY 82 399 IY 400 605 AH 606 228 EL 229 91 HYY 93 522 KYY 524 92 YYA 94 522 KYY 524 92 YYA 94 523 YYG 525 398 YIY 400	ID: Q9TUM0 246 HL 247 588 HL 589 606 HL 607 318 LY 319 399 IY 400 605 AH 606 616 AH 617 228 EL 229 339 EL 340 570 EL 571 91 HYY 93 522 RYY 524 92 YYA 94 523 YYG 525 398 YIY 400 524 YGY 526	ID: Q29477 337 EL 338 37 IR 38 338 LK 339 612 LH 613 246 HL 247 165 AY 166 318 LY 319 81 IY 82 399 IY 400 605 AH 606 228 EL 229 269 EL 270 91 HYY 93 522 KYY 524 92 YYQ 94 523 YYG 525	ID: D3G9G3 612 LH 613 246 HL 247 165 AY 166 318 LY 319 81 IY 82 399 IY 400 605 AH 606 228 EL 229 269 EL 270 91 HYY 93 522 KYY 524 92 YYQ 94 523 YYG 525 398 YIY 400 524 YGY 526 612 LHQ 614

	524 YGY 526 612 LHQ 614 21 RWQ 23 301 KD 302 452 KD 453 7 RW 8 21 RW 22 46 IR 47 328 LK 329 385 QS 386 451 LK 452 672 LK 673 579 KP 580 656 TY 657 465 AGWNIP 470 465 AGWNI 469 467 WNIP 470 466 GWNIP 470 466 GWNI 469 524 YGYTGA 529 594 NHAV 597 447 TW 448 346 VW 347 548 VW 549 652 GGRP 655	210 KD 211 296 KD 297 301 KD 302 544 KD 545 562 KD 563 7 RW 8 328 IR 329 112 LK 113 356 LK 357 385 LK 386 564 LK 565 237 KP 238 282 KP 283 579 KP 580 656 TY 657 64 VY 65 81 VY 82 123 AGWNIP 128 465 AGWNIP 470 123 AGWNI 127 465 AGWNI 469 125 WNIP 128 467 WNIP 470 124 GWNIP 128 466 GWNIP 470 124 GWNI 127 466 GWNI 469 524 YGYTGA529 594 NHAV 597 477 TW 448 346 VW 347 131 LLR 133 307 LLR 309	398 YIY 400 524 YGY 526 612 LHQ 614 296 KD 297 301 KD 302 7 RW 8 46 IR 47 328 LK 329 385 LK 386 451 LK 452 672 LK 673 579 KP 580 656 TY 657 123 AGWNIP 128 465 AGWNIP 470 123 AGWNI 127 465 AGWNI 469 125 WNIP 128 467 WNIP 470 124 GWNIP 128, 466 GWNIP 470 124 GWNI 127 466 GWNI 469 524 YGYTGA 529 594 NHAV 597 447 TW 448 346 VW 347 548 VW 549 270 LLR 272 652 GGRP 655 197 KCL 199 549 WEN 551 193 SGAF 196	296 KD 297 301 KD 302 7 RW 8 46 IR 47 328 LK 329 385 LK 386 451 LK 452 672 LK 673 579 KP 580 656 TY 657 123 AGWNIP 128 465 AGWNIP 470 123 AGWNI 127 465 AGWNI 469 125 WNIP 128 467 WNIP 470 124 GWNIP 128, 466 GWNIP 470 124 GWNI 127 466 GWNI 469 524 YGYTGA 529 594 NHAV 597 447 TW 448 346 VW 347 548 VW 549 270 LLR 272 652 GGRP 655 197 KCL 199 549 WEN 551 193 SGAF 196 629 FC 630 659 KYL 661

		611 LLR 613 140 GPP 142 197 KCL 199 193 SGAF 196 629 FC 630 19 CAQ 21	629 FC 630 659 KYL 661	

Table S2: Potential antidiabetic peptides derived from caseins and whey proteins of camel, cow, goat, and sheep milk predicated by BIOPEP database (http://www.uwn.edu.pI/biochemia/index.php/pI/Biopep)

Protein fraction	Amino acid sequences of antidiabetic peptides			
	Cow milk	Camel milk	Goat milk	Sheep milk
Milk protein:				
Casein:				
α -casein	ID: P02662 ^{196}MP^{197} ^{25}VA^{26} ^{142}LA^{143} ^{26}AP^{27} ^{176}AP^{177} ^{11}LP^{12} ^{72}VP^{73} ^{86}VP^{87} ^{106}VP^{107} ^{112}VP^{113} ^{167}VP^{168} ^{20}LL^{21} ^{98}LL^{99} ^{128}HA^{129} ^{167}VPL^{169} ^{182}IP^{183} ^{28}FP^{29} ^{1}RP^{2} ^{4}HP^{5}	ID: O97943 ^{181}PP^{182} ^{91}MP^{92} ^{211}MP^{212} ^{176}VA^{177} ^{31}LA^{32} ^{108}LL^{109} ^{33}VV^{34} ^{76}VV^{77} ^{151}VV^{152} ^{120}IP^{121} ^{196}TP^{197} ^{35}SP^{36} ^{173}SP^{174} ^{164}FP^{165} ^{1}RP^{2} ^{128}HP^{129} ^{142}HP^{143} ^{190}HP^{191} ^{4}YP^{5}	ID: P18626 ^{196} MP^{197} ^{25}VA^{26} ^{61}KA^{62} ^{142}LA^{143} ^{26}AP^{27} ^{176}AP^{177} ^{128}PA^{129} ^{167}LP^{168} ^{15}VP^{16} ^{72}VP^{73} ^{86}VP^{87} ^{106}VP^{107} ^{112}VP^{113} ^{20}LL^{21} ^{98}LL^{99} ^{24}VV^{25} ^{182}IP^{183} ^{12}SP^{13} ^{28}FP^{29}	ID: P04653 ^{196} MP^{197} ^{25}VA^{26} ^{61}KA^{62} ^{142}LA^{143} ^{26}AP^{27} ^{176}AP^{177} ^{128}PA^{129} ^{167}LP^{168} ^{72}VP^{73} ^{86}VP^{87} ^{106}VP^{107} ^{112}VP^{113} ^{20}LL^{21} ^{98}LL^{99} ^{24}VV^{25} ^{182}IP^{183} ^{28}FP^{29} ^{1}RP^{2} ^{4}HP^{5}

	^{146}YP^{147}	^{8}YP^{9}	^{1}RP^{2}	^{146}YP^{147}
	^{159}YP^{160}	^{180}YP^{181}	^{4}HP^{5}	^{159}YP^{160}
	^{162}GA^{163}	^{171}GA^{172}	^{146}YP^{147}	^{162}GA^{163}
	^{133}EP^{134}	^{188}IA^{189}	^{159}YP^{160}	^{136}IA^{137}
	^{184}NP^{185}	^{200}IA^{201}	^{162}GA^{163}	^{127}NP^{128}
	^{35}EK^{36}	^{15}EP^{16}	^{136}IA^{137}	^{184}NP^{185}
	^{192}EK^{193}	^{53}EP^{54}	^{127}NP^{128}	^{133}QP^{134}
	^{10}GL^{11}	^{147}EP^{148}	^{184}NP^{185}	^{10}GL^{11}
	^{168}PL^{169}	^{162}EP^{163}	^{133}QP^{134}	^{167}LPL^{169}
	^{197}PL^{198}	^{160}HL^{161}	^{10}GL^{11}	^{168}PL^{169}
	^{164}WY^{165}	^{5}PL^{6}	^{167}LPL^{169}	^{197}PL^{198}
	^{198}LW^{199}	^{178}WY^{179}	^{168}PL^{169}	^{164}WY^{165}
	^{163}AW^{164}	^{214}WW^{215}	^{197}PL^{198}	^{198}LW^{199}
	^{173}YT^{174}	^{177}AW^{178}	^{164}WY^{165}	^{163}AW^{164}
	^{62}AE^{63}	^{189}AH^{190}	^{198}LW^{199}	^{173}YT^{174}
	^{116}AE^{117}	^{172}AS^{173}	^{163}AW^{164}	^{76}AE^{77}
	^{143}AY^{144}	^{201}AS^{202}	^{173}YT^{174}	^{116}AE^{117}
	^{158}AY^{159}	^{32}AV^{33}	^{76}AE^{77}	^{62}AG^{63}
	^{51}DQ^{52}	^{157}AY^{158}	^{116}AE^{117}	^{129}AH^{130}
	^{125}EG^{126}	^{140}DN^{141}	^{62}AG^{63}	^{137}AV^{138}
	^{70}EL^{71}	^{116}DQ^{117}	^{129}AH^{130}	^{143}AY^{144}
	^{110}EL^{111}	^{198}EG^{199}	^{137}AV^{138}	^{158}AY^{159}
	^{47}ES^{48}	^{67}ES^{68}	^{143}AY^{144}	^{51}DQ^{52}
	^{63}ES^{64}	^{10}EV^{11}	^{158}AY^{159}	^{125}EG^{126}
	^{14}EV^{15}	^{21}EV^{22}	^{51}DQ^{52}	^{70}EI^{71}
	^{30}EV^{31}	^{75}EV^{76}	^{125}EG^{126}	^{110}EI^{111}
	^{150}FR^{151}	^{12}FQ^{13}	^{70}EI^{71}	^{47}ES^{48}
	^{126}GI^{127}	^{168}FQ^{169}	^{110}EI^{111}	^{14}EV^{15}
	^{137}GV^{138}	^{39}FR^{40}	^{47}ES^{48}	^{30}EV^{31}
	^{93}GY^{94}	^{146}GE^{147}	^{14}EV^{15}	^{32}FR^{33}
	^{80}HI^{81}	^{205}GG^{206}	^{30}EV^{31}	^{150}FR^{151}
	^{121}HS^{122}	^{199}GI^{200}	^{32}FR^{33}	^{93}GY^{94}
	^{127}IH^{128}	^{58}HI^{59}	^{150}FR^{151}	^{121}HS^{122}

	$^{81}IQ^{82}$	$^{101}HR^{102}$	$^{93}GY^{94}$	$^{6}IN^{7}$
	$^{34}KE^{35}$	$^{28}IL^{29}$	$^{8}HR^{9}$	$^{37}IN^{38}$
	$^{83}KE^{84}$	$^{86}IL^{87}$	$^{121}HS^{122}$	$^{81}IQ^{82}$
	$^{124}KE^{125}$	$^{59}IM^{60}$	$^{6}IN^{7}$	$^{34}KE^{35}$
	$^{132}KE^{133}$	$^{137}IN^{138}$	$^{37}IN^{38}$	$^{83}KE^{84}$
	$^{3}KH^{4}$	$^{37}IQ^{38}$	$^{81}IQ^{82}$	$^{124}KE^{125}$
	$^{7}KH^{8}$	$^{114}IR^{115}$	$^{34}KE^{35}$	$^{3}KH^{4}$
	$^{79}KH^{80}$	$^{66}KE^{67}$	$^{83}KE^{84}$	$^{102}KK^{103}$
	$^{102}KK^{103}$	$^{88}KE^{89}$	$^{124}KE^{125}$	$^{114}KS^{115}$
	$^{193}KT^{194}$	$^{27}KI^{28}$	$^{3}KH^{4}$	$^{79}KY^{80}$
	$^{36}KV^{37}$	$^{25}KR^{26}$	$^{102}KK^{103}$	$^{103}KY^{104}$
	$^{105}KV^{106}$	$^{207}KT^{208}$	$^{114}KS^{115}$	$^{120}LH^{121}$
	$^{103}KY^{104}$	$^{150}KV^{151}$	$^{193}KT^{194}$	$^{135}MI^{136}$
	$^{120}LH^{121}$	$^{3}KY^{4}$	$^{79}KY^{80}$	$^{60}MK^{61}$
	$^{16}LN^{17}$	$^{105}KY^{106}$	$^{103}KY^{104}$	$^{123}MK^{124}$
	$^{54}ME^{55}$	$^{100}LH^{101}$	$^{120}LH^{121}$	$^{17}NE^{18}$
	$^{60}ME^{61}$	$^{119}LI^{120}$	$^{135}MI^{136}$	$^{38}NE^{39}$
	$^{135}MI^{136}$	$^{23}LN^{24}$	$^{60}MK^{61}$	$^{19}NL^{20}$
	$^{123}MK^{124}$	$^{103}LN^{104}$	$^{123}MK^{124}$	$^{139}NQ^{140}$
	$^{17}NE^{18}$	$^{60}ME^{61}$	$^{17}NE^{18}$	$^{105}NV^{106}$
	$^{38}NE^{39}$	$^{185}MQ^{186}$	$^{38}NE^{39}$	$^{27}PF^{28}$
	$^{19}NL^{20}$	$^{14}NE^{15}$	$^{7}NH^{8}$	$^{5}PI^{6}$
	$^{139}NQ^{140}$	$^{52}NE^{53}$	$^{19}NL^{20}$	$^{185}PI^{186}$
	$^{27}PF^{28}$	$^{138}NE^{139}$	$^{139}NQ^{140}$	$^{2}PK^{3}$
	$^{5}PI^{6}$	$^{141}NH^{142}$	$^{105}NV^{106}$	$^{113}PK^{114}$
	$^{185}PI^{186}$	$^{163}PF^{164}$	$^{27}PF^{28}$	$^{134}PM^{135}$
	$^{2}PK^{3}$	$^{36}PI^{37}$	$^{5}PI^{6}$	$^{73}PN^{74}$
	$^{134}PM^{135}$	$^{2}PK^{3}$	$^{185}PI^{186}$	$^{183}PN^{184}$
	$^{73}PN^{74}$	$^{143}PQ^{144}$	$^{2}PK^{3}$	$^{107}PQ^{108}$
	$^{113}PN^{114}$	$^{165}PQ^{166}$	$^{113}PK^{114}$	$^{147}PQ^{148}$
	$^{183}PN^{184}$	$^{182}PQ^{183}$	$^{134}PM^{135}$	$^{87}PS^{88}$
	$^{12}PQ^{13}$	$^{212}PQ^{213}$	$^{16}PN^{17}$	$^{160}PS^{161}$
	$^{107}PQ^{108}$	$^{92}PS^{93}$	$^{73}PN^{74}$	$^{177}PS^{178}$

	^{87}PS^{88}	^{191}PS^{192}	^{183}PN^{184}	^{52}QA^{53}
	^{160}PS^{161}	^{54}PT^{55}	^{107}PQ^{108}	^{140}QE^{141}
	^{177}PS^{178}	^{148}PV^{149}	^{147}PQ^{148}	^{152}QF^{153}
	^{52}QA^{53}	^{129}PY^{130}	^{87}PS^{88}	^{97}QL^{98}
	^{13}QE^{14}	^{174}PY^{175}	^{160}PS^{161}	^{108}QL^{109}
	^{140}QE^{141}	^{156}QA^{157}	^{177}PS^{178}	^{119}QL^{120}
	^{152}QF^{153}	^{41}QE^{42}	^{52}QA^{53}	^{148}QL^{149}
	^{9}QG^{10}	^{154}QE^{155}	^{140}QE^{141}	^{155}QL^{156}
	^{97}QL^{98}	^{38}QF^{39}	^{152}QF^{153}	^{172}QY^{173}
	^{108}QL^{109}	^{166}QF^{167}	^{97}QL^{98}	^{9}RG^{10}
	^{155}QL^{156}	^{110}QL^{111}	^{108}QL^{109}	^{33}RK^{34}
	^{130}QQ^{131}	^{133}QL^{134}	^{119}QL^{120}	^{100}RL^{101}
	^{172}QY^{173}	^{144}QL^{145}	^{148}QL^{149}	^{178}SF^{179}
	^{100}RL^{101}	^{169}QL^{170}	^{155}QL^{156}	^{41}SK^{42}
	^{119}RL^{120}	^{13}QN^{14}	^{172}QY^{173}	^{174}TD^{175}
	^{178}SF^{179}	^{183}QV^{184}	^{9}RG^{10}	^{195}TM^{196}
	^{64}SI^{65}	^{213}QW^{214}	^{33}RK^{34}	^{171}TQ^{172}
	^{41}SK^{42}	^{186}QY^{187}	^{100}RL^{101}	^{31}VF^{32}
	^{75}SV^{76}	^{136}RI^{137}	^{178}SF^{179}	^{138}VN^{139}
	^{174}TD^{175}	^{26}RK^{27}	^{41}SK^{42}	^{144}YF^{145}
	^{49}TE^{50}	^{65}RK^{66}	^{174}TD^{175}	^{80}YI^{81}
	^{195}TM^{196}	^{102}RL^{103}	^{49}TE^{50}	^{91}YL^{92}
	^{171}TQ^{172}	^{51}RN^{52}	^{195}TM^{196}	^{94}YL^{95}
	^{194}TT^{195}	^{127}SH^{128}	^{171}TQ^{172}	^{166}YL^{167}
	^{76}VE^{77}	^{18}SI^{19}	^{194}TT^{195}	^{104}YN^{105}
	^{31}VF^{32}	^{193}SY^{194}	^{31}VF^{32}	^{154}YQ^{155}
	^{15}VL^{16}	^{208}TD^{209}	^{138}VN^{139}	^{165}YY^{166}
	^{37}VN^{38}	^{55}TE^{56}	^{144}YF^{145}	^{193}KI^{194}
	^{138}VN^{139}	^{63}TE^{64}	^{80}YI^{81}	^{16}LN^{17}
	^{144}YF^{145}	^{81}TE^{82}	^{91}YL^{92}	^{9}QG^{10}
	^{104}YK^{105}	^{153}TQ^{154}	^{94}YL^{95}	^{48}SI^{49}
	^{91}YL^{92}	^{50}TR^{51}	^{166}YL^{167}	
	^{94}YL^{95}	^{80}TT^{81}	^{104}YN^{105}	

	^{154}YQ^{155} ^{166}YV^{167} ^{165}YY^{166} ^{11}LPQ^{13}	^{11}VF^{12} ^{123}VK^{124} ^{149}VK^{150} ^{22}VL^{23} ^{184}VM^{185} ^{210}VM^{211} ^{34}VS^{35} ^{77}VS^{78} ^{152}VT^{153} ^{194}YD^{195} ^{158}YF^{159} ^{187}YI^{188} ^{106}YK^{107} ^{96}YL^{97} ^{130}YL^{131} ^{135}YR^{136} ^{175}YV^{176} ^{179}YY^{180}	^{154}YQ^{155} ^{165}YY^{166} ^{4}HPINHR^{9}	
B-casein	ID: P02666 ^{64}GP^{65} ^{199}GP^{200} ^{203}GP^{204} ^{75}PP^{76} ^{85}PP^{86} ^{152}PP^{153} ^{158}PP^{159} ^{109}MP^{110} ^{185}MP^{186} ^{102}MA^{103} ^{176}KA^{177} ^{52}FA^{53} ^{103}AP^{104}	ID: Q9TVD0 ^{78}PP^{79} ^{159}PP^{160} ^{110}MP^{111} ^{186}MP^{187} ^{82}PA^{83} ^{72}LP^{73} ^{77}LP^{78} ^{136}LP^{137} ^{138}LP^{139} ^{172}LP^{173} ^{192}LP^{193} ^{86}VP^{87} ^{96}VP^{97}	ID: P33048 ^{64}GP^{65} ^{197}GP^{198} ^{201}GP^{202} ^{85}PP^{86} ^{147}PP^{148} ^{158}PP^{159} ^{109}MP^{110} ^{183}MP^{184} ^{176}KA^{177} ^{52}FA^{53} ^{70}LP^{71} ^{75}LP^{76} ^{135}LP^{136}	ID: P11839 ^{152}PP^{153} ^{151}LP^{152} ^{64}GP^{65} ^{197}GP^{198} ^{201}GP^{202} ^{85}PP^{86} ^{147}PP^{148} ^{158}PP^{159} ^{109}MP^{110} ^{183}MP^{184} ^{176}KA^{177} ^{52}FA^{53} ^{70}LP^{71}

	^{70}LP^{71}	^{118}VP^{119}	^{137}LP^{138}	^{75}LP^{76}
	^{135}LP^{136}	^{152}VP^{153}	^{171}LP^{172}	^{135}LP^{136}
	^{137}LP^{138}	^{174}VP^{175}	^{84}VP^{85}	^{137}LP^{138}
	^{151}LP^{152}	^{179}VP^{180}	^{95}VP^{96}	^{171}LP^{172}
	^{171}LP^{172}	^{198}VP^{199}	^{103}VP^{104}	^{84}VP^{85}
	^{8}VP^{9}	^{208}VP^{209}	^{173}VP^{174}	^{95}VP^{96}
	^{84}VP^{85}	^{213}VP^{214}	^{178}VP^{179}	^{103}VP^{104}
	^{173}VP^{174}	^{112}LL^{113}	^{189}LL^{190}	^{173}VP^{174}
	^{178}VP^{179}	^{140}LL^{141}	^{8}VV^{9}	^{178}VP^{179}
	^{139}LL^{140}	^{163}LL^{164}	^{82}VV^{83}	^{189}LL^{190}
	^{191}LL^{192}	^{117}VV^{118}	^{83}VV^{84}	^{8}VV^{9}
	^{82}VV^{83}	^{67}IP^{68}	^{66}IP^{67}	^{82}VV^{83}
	^{83}VV^{84}	^{104}IP^{105}	^{80}TP^{81}	^{83}VV^{84}
	^{66}IP^{67}	^{148}IP^{149}	^{152}SP^{153}	^{66}IP^{67}
	^{74}IP^{75}	^{158}IP^{159}	^{111}FP^{112}	^{80}TP^{81}
	^{80}TP^{81}	^{155}TP^{156}	^{157}FP^{158}	^{111}FP^{112}
	^{62}FP^{63}	^{115}SP^{116}	^{203}FP^{204}	^{157}FP^{158}
	^{111}FP^{112}	^{53}FP^{54}	^{50}HP^{51}	^{203}FP^{204}
	^{157}FP^{158}	^{206}HP^{207}	^{60}YP^{61}	^{50}HP^{51}
	^{205}FP^{206}	^{69}YP^{70}	^{114}YP^{115}	^{60}YP^{61}
	^{50}HP^{51}	^{181}YP^{182}	^{117}EP^{118}	^{114}YP^{115}
	^{60}YP^{61}	^{216}IA^{217}	^{193}EP^{194}	^{117}EP^{118}
	^{114}YP^{115}	^{184}RA^{185}	^{89}QP^{90}	^{193}EP^{194}
	^{180}YP^{181}	^{65}EP^{66}	^{146}QP^{147}	^{89}QP^{90}
	^{117}EP^{118}	^{196}EP^{197}	^{149}QP^{150}	^{146}QP^{147}
	^{195}EP^{196}	^{8}TA^{9}	^{167}QP^{168}	^{149}QP^{150}
	^{89}QP^{90}	^{55}QP^{56}	^{87}FL^{88}	^{167}QP^{168}
	^{146}QP^{147}	^{81}QP^{82}	^{188}FL^{189}	^{87}FL^{88}
	^{149}QP^{150}	^{90}QP^{91}	^{134}HL^{135}	^{188}FL^{189}
	^{87}FL^{88}	^{150}QP^{151}	^{31}EK^{32}	^{134}HL^{135}
	^{190}FL^{191}	^{210}QP^{211}	^{131}EK^{132}	^{31}EK^{32}
	^{134}HL^{135}	^{76}FL^{77}	^{15}SL^{16}	^{131}EK^{132}
	^{31}EK^{32}	^{88}FL^{89}	^{57}SL^{58}	^{15}SL^{16}

	^{15}SL^{16}	^{135}HL^{136}	^{69}SL^{70}	^{57}SL^{58}
	^{57}SL^{58}	^{2}EK^{3}	^{124}SL^{125}	^{69}SL^{70}
	^{69}SL^{70}	^{32}EK^{33}	^{164}SL^{165}	^{124}SL^{125}
	^{124}SL^{125}	^{12}AL^{13}	^{87}FLQP^{90}	^{164}SL^{165}
	^{164}SL^{165}	^{58}SL^{59}	^{195}VLGP^{198}	^{87}FLQP^{90}
	^{87}FLQP^{90}	^{125}SL^{126}	^{199}VR^{200}	^{195}VLGP^{198}
	^{197}VLGP^{200}	^{143}SL^{144}	^{75}LPL^{77}	^{199}VR^{200}
	^{20}1VR^{20}	^{162}SL^{163}	^{135}LPL^{137}	^{75}LPL^{77}
	^{135}LPL^{137}	^{165}SL^{166}	^{137}LPL^{139}	^{135}LPL^{137}
	^{137}LPL^{139}	^{204}GL^{205}	^{135}LPLPL^{139}	^{137}LPL^{139}
	^{135L}PLPL^{139}	^{88F}LQ^{P91}	^{76}PL^{77}	^{135}LPLPL^{139}
	^{76}PL^{77}	^{202}VR^{203}	^{136}PL^{137}	^{76}PL^{77}
	^{136}PL^{137}	^{136}LPL^{138}	^{138}PL^{139}	^{136}PL^{137}
	^{138}PL^{139}	^{138}LPL^{140}	^{150}PL^{151}	^{138}PL^{139}
	^{150}PL^{151}	^{136L}PLPL^{140}	^{143}WM^{144}	^{150}PL^{151}
	^{75}PPL^{77}	^{79}PL^{80}	^{88}LQP^{90}	^{143}WM^{144}
	^{70}LPQNIPP^{76}	^{111}PL^{112}	^{187}AF^{188}	^{88}LQP^{90}
	^{62}FPGPIPN^{68}	^{137}PL^{138}	^{177}AV^{178}	^{187}AF^{188}
	^{74}IPPLTQTPV^{82}	^{139}PL^{140}	^{91}EI^{92}	^{177}AV^{178}
	^{143}WM^{144}	^{211}PL^{212}	^{14}ES^{15}	^{91}EI^{92}
	^{88}LQP^{90}	^{78}PPL^{80}	^{21}ES^{22}	^{14}ES^{15}
	^{189}AF^{190}	^{80}LQP^{82}	^{121}ES^{122}	^{21}ES^{22}
	^{177}AV^{178}	^{89}LQP^{91}	^{11}ET^{12}	^{121}ES^{122}
	^{11}EI^{12}	^{51}YT^{52}	^{100}ET^{101}	^{11}ET^{12}
	^{14}ES^{15}	^{9}AG^{10}	^{33}FQ^{34}	^{100}ET^{101}
	^{21}ES^{22}	^{83}AV^{84}	^{10}GE^{11}	^{33}FQ^{34}
	^{121}ES^{122}	^{190}AV^{191}	^{94}GV^{95}	^{10}GE^{11}
	^{91}EV^{92}	^{200}DP^{201}	^{25}HI^{26}	^{94}GV^{95}
	^{33}FQ^{34}	^{14}ES^{15}	^{49}IH^{50}	^{25}HI^{26}
	^{10}GE^{11}	^{21}ES^{22}	^{74}IL^{75}	^{49}IH^{50}
	^{94}GV^{95}	^{122}ES^{123}	^{205}IL^{206}	^{74}IL^{75}
	^{49}IH^{50}	^{101}ET^{102}	^{92}IM^{93}	^{205}IL^{206}
	^{207}II^{208}	^{194}FQ^{195}	^{26}IN^{27}	^{92}IM^{93}

	^{26}IN^{27}	^{10}GE^{11}	^{185}IQ^{186}	^{26}IN^{27}
	^{187}IQ^{188}	^{25}HI^{26}	^{99}KE^{100}	^{185}IQ^{186}
	^{99}KE^{100}	^{63}HT^{64}	^{107}KE^{108}	^{99}KE^{100}
	^{107}KE^{108}	^{103}II^{104}	^{32}KF^{33}	^{107}KE^{108}
	^{32}KF^{33}	^{71}IL^{72}	^{105}KH^{106}	^{32}KF^{33}
	^{105}KH^{106}	^{26}IN^{27}	^{29}KI^{30}	^{105}KH^{106}
	^{29}KI^{30}	^{3}KE^{4}	^{48}KI^{49}	^{29}KI^{30}
	^{48}KI^{49}	^{100}KE^{101}	^{28}KK^{29}	^{48}KI^{49}
	^{28}KK^{29}	^{108}KE^{109}	^{97}KV^{98}	^{28}KK^{29}
	^{97}KV^{98}	^{33}KF^{34}	^{169}KV^{170}	^{97}KV^{98}
	^{169}KV^{170}	^{30}KI^{31}	^{113}KY^{114}	^{169}KV^{170}
	^{113}KY^{114}	^{35}KI^{36}	^{133}LH^{134}	^{113}KY^{114}
	^{133}LH^{134}	^{49}KI^{50}	^{6}LN^{7}	^{133}LH^{134}
	^{6}LN^{7}	^{106}KR^{107}	^{77}LT^{78}	^{6}LN^{7}
	^{77}LT^{78}	^{7}KT^{8}	^{125}LT^{126}	^{77}LT^{78}
	^{125}LT^{126}	^{98}KT^{99}	^{127}LT^{128}	^{125}LT^{126}
	^{127}LT^{128}	^{92}KV^{93}	^{58}LV^{59}	^{127}LT^{128}
	^{58}LV^{59}	^{170}KV^{171}	^{139}LV^{140}	^{58}LV^{59}
	^{156}MF^{157}	^{134}LH^{135}	^{206}LV^{207}	^{139}LV^{140}
	^{93}MG^{94}	^{205}LH^{206}	^{156}MF^{157}	^{206}LV^{207}
	^{144}MH^{145}	^{144}LM^{145}	^{93}MG^{94}	^{156}MF^{157}
	^{132}NL^{133}	^{126}LT^{127}	^{144}MH^{145}	^{93}MG^{94}
	^{7}NV^{8}	^{128}LT^{129}	^{102}MV^{103}	^{144}MH^{145}
	^{51}PF^{52}	^{59}LV^{60}	^{7}NV^{8}	^{102}MV^{103}
	^{61}PF^{62}	^{212}LV^{213}	^{51}PF^{52}	^{7}NV^{8}
	^{86}PF^{87}	^{157}MI^{158}	^{61}PF^{62}	^{51}PF^{52}
	^{110}PF^{111}	^{85}MV^{86}	^{86}PF^{87}	^{61}PF^{62}
	^{118}PF^{119}	^{178}MV^{179}	^{110}PF^{111}	^{86}PF^{87}
	^{204}PF^{205}	^{14}MY^{146}	^{118}PF^{119}	^{110}PF^{111}
	^{9}PG^{10}	^{75}NF^{76}	^{202}PF^{203}	^{118}PF^{119}
	^{63}PG^{64}	^{133}NL^{134}	^{65}PI^{66}	^{202}PF^{203}
	^{147}PH^{148}	^{87}PF^{88}	^{184}PI^{185}	^{65}PI^{66}
	^{65}PI^{66}	^{119}PF^{120}	^{204}PI^{205}	^{184}PI^{185}

	^{186}PI^{187}	^{193}PF^{194}	^{96}PK^{97}	^{204}PI^{205}
	^{206}PI^{207}	^{66}PI^{67}	^{104}PK^{105}	^{96}PK^{97}
	^{104}PK^{105}	^{70}PI^{71}	^{112}PK^{113}	^{104}PK^{105}
	^{112}PK^{113}	^{91}PK^{92}	^{168}PK^{169}	^{112}PK^{113}
	^{67}PN^{68}	^{97}PK^{98}	^{67}PN^{68}	^{168}PK^{169}
	^{71}PQ^{72}	^{105}PK^{106}	^{71}PQ^{72}	^{67}PN^{68}
	^{159}PQ^{160}	^{156}PM^{157}	^{148}PQ^{149}	^{71}PQ^{72}
	^{174}PQ^{175}	^{54}PQ^{55}	^{159}PQ^{160}	^{148}PQ^{149}
	^{181}PQ^{182}	^{56}PQ^{57}	^{174}PQ^{175}	^{159}PQ^{160}
	^{153}PT^{154}	^{73}PQ^{74}	^{179}PQ^{180}	^{174}PQ^{175}
	^{81}PV^{82}	^{149}PQ^{150}	^{153}PT^{154}	^{179}PQ^{180}
	^{115}PV^{116}	^{153}PQ^{154}	^{81}PV^{82}	^{153}PT^{154}
	^{172}PV^{173}	^{160}PQ^{161}	^{115}PV^{116}	^{81}PV^{82}
	^{196}PV^{197}	^{175}PQ^{176}	^{172}PV^{173}	^{115}PV^{116}
	^{200}PV^{201}	^{182}PQ^{183}	^{194}PV^{195}	^{172}PV^{173}
	^{179}PY^{180}	^{209}PQ^{210}	^{198}PV^{199}	^{194}PV^{195}
	^{188}QA^{189}	^{116}PV^{117}	^{54}QA^{55}	^{198}PV^{199}
	^{46}QD^{47}	^{151}PV^{152}	^{186}QA^{187}	^{54}QA^{55}
	^{194}QE^{195}	^{173}PV^{174}	^{46}QD^{47}	^{186}QA^{187}
	^{72}QN^{73}	^{187}PV^{188}	^{3}QE^{4}	^{46}QD^{47}
	^{38}QQ^{39}	^{197}PV^{198}	^{192}QE^{193}	^{3}QE^{4}
	^{39}QQ^{40}	^{201}PV^{202}	^{72}QN^{73}	^{192}QE^{193}
	^{34}QS^{35}	^{207}PV^{208}	^{38}QQ^{39}	^{72}QN^{73}
	^{56}QS^{57}	^{214}PV^{215}	^{39}QQ^{40}	^{38}QQ^{39}
	^{123}QS^{124}	^{68}PY^{69}	^{34}QS^{35}	^{39}QQ^{40}
	^{141}QS^{142}	^{180}PY^{181}	^{56}QS^{57}	^{34}QS^{35}
	^{160}QS^{161}	^{189}QA^{190}	^{123}QS^{124}	^{56}QS^{57}
	^{167}QS^{168}	^{47}QD^{48}	^{141}QS^{142}	^{123}QS^{124}
	^{40}QT^{41}	^{195}QE^{196}	^{160}QS^{161}	^{141}QS^{142}
	^{54}QT^{55}	^{168}QF^{169}	^{40}QT^{41}	^{160}QS^{161}
	^{79}QT^{80}	^{147}QI^{148}	^{79}QT^{80}	^{40}QT^{41}
	^{202}RG^{203}	^{74}QN^{75}	^{200}RG^{201}	^{79}QT^{80}
	^{25}RI^{26}	^{39}QQ^{40}	^{22}SI^{23}	^{200}RG^{201}

	^{22}SI^{23} ^{96}SK^{97} ^{168}SK^{169} ^{161}SV^{162} ^{142}SW^{143} ^{128}TD^{129} ^{41}TE^{42} ^{120}TE^{121} ^{126}TL^{127} ^{55}TQ^{56} ^{78}TQ^{79} ^{24}TR^{25} ^{154}TV^{155} ^{13}VE^{14} ^{116}VE^{117} ^{130}VE^{131} ^{98}VK^{99} ^{162}VL^{163} ^{170}VL^{171} ^{197}VL^{198} ^{92}VM^{93} ^{155}VM^{156} ^{95}VS^{96} ^{59}VY^{60} ^{193}YQ^{194} ^{71}PQNIPPL^{77} ^{199}GPV^{201} ^{70}LPQNIPPL^{77} ^{114}YPVEPF^{119} ^{70}LPQ^{72}	^{40}QQ^{41} ^{46}QQ^{47} ^{176}QQ^{177} ^{57}QS^{58} ^{114}QS^{115} ^{124}QS^{125} ^{142}QS^{143} ^{161}QS^{162} ^{41}QT^{42} ^{154}QT^{155} ^{203}RG^{204} ^{107}RK^{108} ^{62}SH^{63} ^{15}SI^{16} ^{22}SI^{23} ^{129}TD^{130} ^{42}TE^{43} ^{64}TE^{65} ^{121}TE^{122} ^{52}TF^{53} ^{24}TH^{25} ^{102}TI^{103} ^{99}TK^{100} ^{127}TL^{128} ^{215}VI^{216} ^{171}VL^{172} ^{191}VL^{192} ^{84}VM^{85} ^{93}VM^{94} ^{188}VQ^{189} ^{60}VY^{61} ^{146}YQ^{147} ^{61}YS^{62}	^{161}SV^{162} ^{142}SW^{143} ^{128}TD^{129} ^{41}TE^{42} ^{120}TE^{121} ^{63}TG^{64} ^{24}TH^{25} ^{126}TL^{127} ^{101}TM^{102} ^{78}TQ^{79} ^{12}TV^{13} ^{154}TV^{155} ^{13}VE^{14} ^{116}VE^{117} ^{130}VE^{131} ^{9}VG^{10} ^{98}VK^{99} ^{162}VL^{163} ^{170}VL^{171} ^{195}VL^{196} ^{155}VM^{156} ^{140}VQ^{141} ^{59}VY^{60} ^{191}YQ^{192} ^{197}GPV^{199} ^{201}GPFPILV^{207} ^{114}YPVEPF^{119} ^{70}LPQ^{72}	^{22}SI^{23} ^{161}SV^{162} ^{142}SW^{143} ^{128}TD^{129} ^{41}TE^{42} ^{120}TE^{121} ^{63}TG^{64} ^{24}TH^{25} ^{126}TL^{127} ^{101}TM^{102} ^{78}TQ^{79} ^{12}TV^{13} ^{154}TV^{155} ^{13}VE^{14} ^{116}VE^{117} ^{130}VE^{131} ^{9}VG^{10} ^{98}VK^{99} ^{162}VL^{163} ^{170}VL^{171} ^{195}VL^{196} ^{155}VM^{156} ^{140}VQ^{141} ^{59}VY^{60} ^{191}YQ^{192} ^{197}GPV^{199} ^{201}GPFPILV^{207} ^{114}YPVEPF^{119} ^{70}LPQ^{72}

		^{72}LPQ^{74}		
K-casein	ID: P02668 ^{25}IP^{127} ^{108}PP^{109} ^{155}PP^{156} ^{47}VA^{48} ^{142}VA^{143} ^{94}MA^{95} ^{105}MA^{106} ^{63}PA^{64} ^{69}PA^{70} ^{83}PA^{84} ^{55}LP^{56} ^{82}VP^{83} ^{25}IP^{26} ^{107}IP^{108} ^{118}IP^{119} ^{132}TP^{133} ^{68}SP^{69} ^{148}SP^{149} ^{154}SP^{155} ^{45}KP^{46} ^{62}KP^{63} ^{97}HP^{98} ^{99}HP^{100} ^{34}YP^{35} ^{57}YP^{58} ^{21}LA^{22} ^{124}LA^{125} ^{128}EP^{129} ^{166}TA^{167} ^{6}QP^{7} ^{90}QP^{91}	ID: P79139 ^{84}PP^{85} ^{101}PP^{102} ^{122}PP^{123} ^{65}VA^{66} ^{117}VA^{118} [^{47}LA^{48} ^{112}PA^{113} ^{130}PA^{131} ^{79}LP^{80} ^{49}VP^{50} ^{17}LL^{18} ^{135}VV^{136} ^{70}HA^{71} ^{56}IP^{57} ^{73}IP^{74} ^{100}IP^{101} ^{125}IP^{126} ^{149}TP^{150} ^{26}FP^{27} ^{90}RP^{91} ^{92}RP^{93} ^{94}RP^{95} ^{63}KP^{64} ^{35}YP^{36} ^{58}YP^{59} ^{98}IA^{99} ^{137}IA^{138} ^{121}EP^{122} ^{129}EP^{130} ^{111}NP^{112} ^{127}TA^{128}	ID: P02670 ^{25}IP^{127} ^{108}PP^{109} ^{47}VA^{48} ^{64}VA^{65} ^{105}MA^{106} ^{94}LA^{95} ^{69}PA^{70} ^{83}PA^{84} ^{119}PA^{120} ^{55}LP^{56} ^{78}LP^{79} ^{82}VP^{83} ^{118}VP^{119} ^{25}IP^{26} ^{107}IP^{108} ^{134}TP^{135} ^{68}SP^{69} ^{45}RP^{46} ^{62}KP^{63} ^{97}HP^{98} ^{99}HP^{100} ^{34}YP^{35} ^{57}YP^{58} ^{21}IA^{22} ^{124}IA^{125} ^{154}IA^{155} ^{128}EP^{129} ^{146}NP^{147} ^{162}TA^{163} ^{6}Q^{P7} ^{90}QP^{91}	ID: P02669 ^{25}IP^{127} ^{108}PP^{109} ^{47}VA^{48} ^{64}VA^{65} ^{105}MA^{106} ^{94}MA^{95} ^{157}AP^{158} ^{140}VV^{141} ^{118}IPA^{120} ^{118}IP^{119} ^{92}TA^{93} ^{81}AV^{82} ^{139}AV^{140} ^{143}AV^{144} ^{117}EI^{118} ^{69}PA^{70} ^{83}PA^{84} ^{119}PA^{120} ^{55}LP^{56} ^{78}LP^{79} ^{82}VP^{83} ^{80}NA^{81} ^{142}NA^{143} ^{7}RI^{8} ^{25}IP^{26} ^{107}IP^{108} ^{134}TP^{135} ^{68}SP^{69} ^{45}RP^{46} ^{62}KP^{63} ^{97}HP^{98}

	^{54}FL^{55}	^{7}QP^{8}	^{54}FL^{55}	^{99}HP^{100}
	^{101}HL^{102}	^{12}EK^{13}	^{10}1HL^{102}	^{34}YP^{35}
	^{11}EK^{12}	^{20}EK^{21}	^{11}EK^{12}	^{57}YP^{58}
	^{48}AL^{49}	^{78}AL^{79}	^{48}AL^{49}	^{21}IA^{22}
	^{38}GL^{39}	^{128}AE^{129}	^{38}GL^{39}	^{124}IA^{125}
	^{66}VR^{67}	^{138}AE^{139}	^{66}VR^{67}	^{154}IA^{155}
	^{25}IPIQY^{29}	^{140}AS^{141}	^{25}IPIQY^{29}	^{128}EP^{129}
	^{55}LPYPY^{59}	^{118}AT^{119}	^{55}LPYPY^{59}	^{146}NP^{147}
	^{57}YPYY^{60}	^{48}AV^{49}	^{57}YPYY^{60}	^{162}TA^{163}
	^{57}YPY^{59}	^{131}AV^{132}	^{57}YPY^{59}	^{90}QP^{91}
	^{64}AA^{65}	^{83}DP^{84}	^{75}WQ^{76}	^{54}FL^{55}
	^{75}WQ^{76}	^{161}EI^{162}	^{127}AE^{128}	^{10}1HL^{102}
	^{125}AS^{126}	^{151}ET^{152}	^{125}AS^{126}	^{11}EK^{12}
	^{143}AT^{144}	^{1}EV^{2}	^{149}AS^{150}	^{48}AL^{49}
	^{65}AV^{66}	^{39}GI^{40}	^{155}AS^{156}	^{38}GL^{39}
	^{137}AV^{138}	^{45}HR^{46}	^{157}AS^{158}	^{66}VR^{67}
	^{167}AV^{168}	^{40}IN^{41}	^{65}AV^{66}	^{25}IPIQY^{29}
	^{117}EI^{118}	^{51}IN^{52}	^{145}DN^{146}	^{55}LPYPY^{59}
	^{157}EI^{158}	^{114}IN^{115}	^{89}DQ^{90}	^{57}YPYY^{60}
	^{139}ES$^{140]}$	^{28}IQ^{29}	^{112}DQ^{113}	^{57}YPY^{59}
	^{153}ES^{154}	^{67}IR^{68}	^{152}ES^{153}	^{75}WQ^{76}
	^{15}0EV^{151}	^{103}KK^{104}	^{159}ET^{160}	^{127}AE^{128}
	^{127}GE^{128}	^{21}KT^{22}	^{117}EV^{118}	^{125}AS^{126}
	^{72}IL^{73}	^{104}KT^{105}	^{169}EV^{170}	^{149}AS^{150}
	^{50}IN^{51}	^{108}KT^{109}	^{132}HS^{133}	^{155}AS^{156}
	^{121}IN^{122}	^{13}KV^{14}	^{50}IN^{51}	^{65}AV^{66}
	^{158}IN^{159}	^{24}KY^{25}	^{121}IN^{122}	^{145}DN^{146}
	^{27}IQ^{28}	^{69}LH^{70}	^{27}IQ^{28}	^{89}DQ^{90}
	^{8}IR^{9}	^{18}LN^{19}	^{20}KI^{21}	^{112}DQ^{113}
	^{20}KI^{21}	^{19}NE^{20}	^{110}KK^{111}	^{152}ES^{153}
	^{110}KK^{111}	^{52}NN^{53}	^{85}KS^{86}	^{159}ET^{160}
	^{85}KS^{86}	^{4}NQ^{5}	^{115}KT^{116}	^{169}EV^{170}
	^{115}KT^{116}	^{53}NQ^{54}	^{23}KY^{24}	^{132}HS^{133}

	^{23}KY^{24}	^{115}NT^{116}	^{49}LI^{50}	^{50}IN^{51}
	^{49}LI^{50}	^{133}NT^{134}	^{39}LN^{40}	^{121}IN^{122}
	^{39}LN^{40}	^{41}NY^{42}	^{51}NN^{52}	^{27}IQ^{28}
	^{51}NN^{52}	^{60}NY^{61}	^{3}NQ^{4}	^{20}KI^{21}
	^{3}NQ^{4}	^{27}PI^{28}	^{52}NQ^{53}	^{110}KK^{111}
	^{52}NQ^{53}	^{50}PI^{51}	^{80}NT^{81}	^{85}KS^{86}
	^{112}NQ^{113}	^{102}PK^{103}	^{142}NT^{143}	^{115}KT^{116}
	^{80}NT^{81}	^{59}PN^{60}	^{7}PI^{8}	^{23}KY^{24}
	^{122}NT^{123}	^{80}PN^{81}	^{122}NT^{123}	^{49}LI^{50}
	^{159}NT^{160}	^{74}PQ^{75}	^{161}NT1^{62}	^{39}LN^{40}
	^{40}NY^{41}	^{36}PS^{37}	^{40}NY^{41}	^{51}NN^{52}
	^{98}PH^{99}	^{95}PS^{96}	^{98}PH^{99}	^{3}NQ^{4}
	^{100}PH^{101}	^{8}PT^{9}	^{100}PH^{101}	^{52}NQ^{53}
	^{7}PI^{8}	^{85}PT^{86}	^{26}PI^{27}	^{122}NT^{123}
	^{26}PI^{27}	^{126}PT^{127}	^{109}PK^{110}	^{161}NT1^{62}
	^{109}PK^{110}	^{64}PV^{65}	^{79}PN^{80}	^{40}NY^{41}
	^{35}PS^{36}	^{123}PV^{124}	^{35}PS^{36}	^{98}PH^{99}
	^{91}PT^{92}	^{57}PY^{58}	^{91}PT^{92}	^{100}PH^{101}
	^{119}PT^{120}	^{77}QA^{78}	^{129}PT^{130}	^{26}PI^{27}
	^{129}PT^{130}	^{106}QD^{107}	^{135}PT^{136}	^{109}PK^{110}
	^{133}PT^{134}	^{5}QE^{6}	^{46}PV^{47}	^{79}PN^{80}
	^{46}PV^{47}	^{29}QF^{30}	^{63}PV^{64}	^{35}PS^{36}
	^{56}PY^{57}	^{54}QF^{55}	^{56}PY^{57}	^{91}PT^{92}
	^{58}PY^{59}	^{44}QH^{45}	^{58}PY^{59}	^{129}PT^{130}
	^{88}QA^{89}	^{72}QI^{73}	^{88}QD^{89}	^{135}PT^{136}
	^{113}QD^{114}	^{156}QI^{157}	^{113}QD^{114}	^{46}PV^{47}
	^{4}QE^{5}	^{3}QN^{4}	^{4}QE^{5}	^{63}PV^{64}
	^{53}QF^{54}	^{32}QS^{33}	^{53}QF^{54}	^{56}PY^{57}
	^{71}QI^{72}	^{16}RL^{17}	^{2}QN^{3}	^{58}PY^{59}
	^{2}QN^{3}	^{46}RL^{47}	^{43}QQ^{44}	^{88}QD^{89}
	^{43}QQ^{44}	^{68}RL^{69}	^{71}QT^{72}	^{113}QD^{114}
	^{76}QV^{77}	^{89}RR^{90}	^{76}QV^{77}	^{4}QE^{5}
	^{162}QV^{163}	^{96}SF^{97}	^{164}QV^{165}	^{53}QF^{54}

	^{74}QW^{75} ^{28}QY^{29} ^{96}RH^{97} ^{103}SF^{104} ^{36}SY^{37} ^{116}TE^{117} ^{135}TE^{136} ^{120}TI^{121} ^{123}TI^{124} ^{144}TL^{145} ^{93}TM^{94} ^{130}TS^{131} ^{164}TS^{165} ^{92}TT^{93} ^{134}TT^{135} ^{81}TV^{82} ^{141}TV^{142} ^{160}TV^{161} ^{138}VE^{139} ^{151}VI^{152} ^{30}VL^{31} ^{77}VL^{78} ^{161}VQ^{162} ^{163}VT^{164} ^{60}YA^{61} ^{37}YG^{38} ^{24}YI^{25} ^{42}YQ^{43} ^{29}YV^{30} ^{41}YY^{42} ^{59}YY^{60} ^{50}INNQFLPYPY^{59}	^{37}SY^{38} ^{160}TE^{161} ^{105}TQ^{106} ^{147}TS^{148} ^{158}TS^{159} ^{146}TT^{147} ^{152}TT^{153} ^{153}TT^{154} ^{22}TV^{23} ^{86}TV^{87} ^{109}TV^{110} ^{116}TV^{117} ^{119}TV^{120} ^{134}TV^{135} ^{154}TV^{155} ^{14}VE^{15} ^{87}VE^{88} ^{120}VE^{121} ^{124}VI^{125} ^{136}VI^{137} ^{23}VK^{24} ^{110}VN^{111} ^{132}VN^{133} ^{2}VQ^{3} ^{31}VQ^{32} ^{155}VQ^{156} ^{61}YA^{62} ^{25}YF^{26} ^{38}YG^{39} ^{43}YQ^{44} ^{42}YY^{43}	^{74}QW^{75} ^{28}QY^{29} ^{96}RH^{97} ^{103}SF^{104} ^{153}SI^{154} ^{36}SY^{37} ^{116}TE^{117} ^{137}TE^{138} ^{168}TE^{169} ^{123}TI^{124} ^{72}TL^{73} ^{160}TN^{161} ^{166}TS1^{67} ^{136}TT^{137} ^{130}TV^{131} ^{144}VD^{145} ^{131}VH^{132} ^{30}VL^{31} ^{77}VL^{78} ^{141}VN^{142} ^{165}VT^{166} ^{60}YA^{61} ^{37}YG^{38} ^{24}YI^{25} ^{42}YQ^{43} ^{29}YV^{30} ^{41}YY^{42} ^{59}YY^{60} ^{50}INNQFLPYPY^{59} ^{143}TV^{144} ^{93}TL^{94} ^{92}TT^{93} ^{81}TV^{82}	^{2}QN^{3} ^{43}QQ^{44} ^{71}QT^{72} ^{76}QV^{77} ^{164}QV^{165} ^{74}QW^{75} ^{28}QY^{29} ^{96}RH^{97} ^{103}SF^{104} ^{153}SI^{154} ^{36}SY^{37} ^{116}TE^{117} ^{137}TE^{138} ^{168}TE^{169} ^{123}TI^{124} ^{72}TL^{73} ^{160}TN^{161} ^{166}TS1^{67} ^{136}TT^{137} ^{130}TV^{131} ^{144}VD^{145} ^{131}VH^{132} ^{30}VL^{31} ^{77}VL^{78} ^{141}VN^{142} ^{165}VT^{166} ^{60}YA^{61} ^{37}YG^{38} ^{24}YI^{25} ^{42}YQ^{43} ^{29}YV^{30} ^{41}YY^{42} ^{59}YY^{60}

				^{50}INNQFLPYPY^{59}
GMP	^{4}PP^{5}	^{4}PP^{5}	^{14}VP^{15}	^{53}AP^{54}
	^{51}PP^{52}	^{25}PP^{26}	^{53}AS^{54}	^{36}VV^{37}
	^{38}VA^{39}	^{20}VA^{21}	^{13}EV^{14}	^{14}IPA^{16}
	^{1}MA^{2}	^{15}PA^{16}	^{38}NT^{39}	^{14}IP^{15}
	^{3}IP^{4}	^{33}PA^{34}	^{39}TV^{40}	^{38}NA^{39}
	^{14}IP^{15}	^{38}VV^{39}	^{4}PP^{5}	^{35}AV^{36}
	^{28}TP^{29}	^{3}IP^{4}	^{1}MA^{2}	^{39}AV^{40}
	^{44}SP^{45}	^{28}IP^{29}	^{15}PA^{16}	^{13}EI^{14}
	^{50}SP^{51}	^{52}TP^{53}	^{3}IP^{4}	^{4}PP^{5}
	^{20}IA^{21}	^{1}IA^{2}	^{30}TP^{31}	^{1}MA^{2}
	^{24}EP^{25}	^{40}IA^{41}	^{20}IA^{21}	^{15}PA^{16}
	^{62}TA^{63}	^{24}EP^{25}	^{50}IA^{51}	^{3}IP^{4}
	^{21}AS^{22}	^{32}EP^{33}	^{24}EP^{25}	^{30}TP^{31}
	^{39}AT^{40}	^{14}NP^{15}	^{42}NP^{43}	^{20}IA^{21}
	^{33}AV^{34}	^{30}TA^{31}	^{59}TA^{60}	^{50}IA^{51}
	^{63}AV^{64}	^{31}AE^{32}	^{23}AE^{24}	^{24}EP^{25}
	^{13}EI^{14}	^{41}AE^{42}	^{21}AS^{22}	^{42}NP^{43}
	^{53}EI^{54}	^{43}AS^{44}	^{45}AS^{46}	^{59}TA^{60}
	^{35}ES^{36}	^{21}AT^{22}	^{51}AS^{52}	^{23}AE^{24}
	^{49}ES^{50}	^{34}AV^{35}	^{41}DN^{42}	^{21}AS^{22}
	^{46}EV^{47}	^{64}EI^{65}	^{8}DQ^{9}	^{45}AS^{46}
	^{23}GE^{24}	^{54}ET^{55}	^{48}ES^{49}	^{51}AS^{52}
	^{17}IN^{18}	^{17}IN^{18}	^{56}ET^{57}	^{41}DN^{42}
	^{54}IN^{55}	^{6}KK^{7}	^{66}EV^{67}	^{8}DQ^{9}
	^{6}KK^{7}	^{7}KT^{8}	^{28}HS^{29}	^{48}ES^{49}
	^{11}KT^{12}	^{11}KT^{12}	^{17}IN^{18}	^{56}ET^{57}
	^{8}NQ^{9}	^{18}NT^{19}	^{6}KK^{7}	^{66}EV^{67}
	^{18}NT^{19}	^{36}NT^{37}	^{11}KT^{12}	^{28}HS^{29}
	^{55}NT^{56}	^{5}PK^{6}	^{18}NT^{19}	^{17}IN^{18}
	^{5}PK^{6}	^{29}PT^{30}	^{58}NT^{59}	^{6}KK^{7}
	^{15}PT^{16}	^{26}PV^{27}	^{5}PK^{6}	^{11}KT^{12}
	^{25}PT^{26}	^{9}QD^{10}	^{25}PT^{26}	^{18}NT^{19}

	^{29}PT^{30} ^{9}QD^{10} ^{58}QV^{59} ^{12}TE^{13} ^{31}TE^{32} ^{16}TI^{17} ^{19}TI^{20} ^{40}TL^{41} ^{26}TS^{27} ^{60}TS^{61} ^{30}TT^{31} ^{37}TV^{38} ^{56}TV^{57} ^{34}VE^{35} ^{47}VI^{48} ^{57}VQ^{58} ^{59}VT^{60}	^{59}QI^{60} ^{63}TE^{64} ^{8}TQ^{9} ^{50}TS^{51} ^{61}TS^{62} ^{49}TT^{50} ^{55}TT^{56} ^{56}TT^{57} ^{12}TV^{13} ^{19}TV^{20} ^{22}TV^{23} ^{37}TV^{38} ^{57}TV^{58} ^{23}VE^{24} ^{27}VI^{28} ^{39}VI^{40} ^{13}VN^{14} ^{35}VN^{36} ^{58}VQ^{59}	^{31}PT^{32} ^{9}QD^{10} ^{61}QV^{62} ^{49}SI^{50} ^{12}TE^{13} ^{33}TE^{34} ^{65}TE^{66} ^{19}TI^{20} ^{57}TN^{58} ^{63}TS^{64} ^{32}TT^{33} ^{26}TV^{27} ^{40}VD^{41} ^{27}VH^{28} ^{37}VN^{38} ^{62}VT^{63}	^{58}NT^{59} ^{5}PK^{6} ^{25}PT^{26} ^{31}PT^{32} ^{9}QD^{10} ^{61}QV^{62} ^{49}SI^{50} ^{12}TE^{13} ^{33}TE^{34} ^{65}TE^{66} ^{19}TI^{20} ^{57}TN^{58} ^{63}TS^{64} ^{32}TT^{33} ^{26}TV^{27} ^{40}VD^{41} ^{27}VH^{28} ^{37}VN^{38} ^{62}VT^{63}
Whey protein				
A-lactalbumin	ID: P00711 ^{108}KA^{109} ^{105}LA^{106} ^{23}LP^{24} ^{66}NP^{67} ^{80}FL^{81} ^{26}WV^{27} ^{113}EK^{114} ^{121}EK^{122} ^{109}AL^{110} ^{22}SL^{23} ^{51}GL^{52} ^{23}LPEWVCTTFH^{32}	ID: P00710 ^{23}LA^{24} ^{105}LA^{106} ^{41}VV^{42} ^{108}KP^{109} ^{80}FL^{81} ^{113}EK^{114} ^{121}EK^{122} ^{51}GL^{52} ^{109}PL^{110} ^{104}WL^{105} ^{118}WQ^{119} ^{26}WI^{27}	ID: P00712 ^{108}KA^{109} ^{105}LA^{106} ^{23}LP^{24} ^{66}NP^{67} ^{29}TA^{30} ^{80}FL^{81} ^{26}WV^{27} ^{113}EK^{114} ^{121}EK^{122} ^{109}AL^{110} ^{22}SL^{23} ^{51}GL^{52}	ID: P09462 ^{89}IM^{90} ^{10}QE^{11} ^{92}VK^{93} ^{108}KA^{109} ^{105}LA^{106} ^{23}LP^{24} ^{66}NP^{67} ^{29}TA^{30} ^{80}FL^{81} ^{26}WV^{27} ^{113}EK^{114} ^{121}EK^{122}

	^{105}LAHKALCSEK^{114} ^{60}WCKDDQNPHS^{69} ^{110}LCSEKLDQWL^{119} ^{1}EQLTKCEVFR^{10} ^{8}VFRELKDLKG^{17} ^{17}GYGGVSLPEW^{26} ^{31}FHTSGYDTQA^{40} ^{33}TSGYDTQAIV^{42} ^{37}DTQAIVQNND^{46} ^{61}CKDDQNPHSS^{70} ^{65}QNPHSSNICN^{74} ^{104}WL^{105} ^{118}WL^{119} ^{60}WC^{61} ^{106}AH^{107} ^{64}DQ^{65} ^{116}DQ^{117} ^{7}EV^{8} ^{25}EW^{26} ^{49}EY^{50} ^{53}FQ^{54} ^{9}FR^{10} ^{19}GG^{20} ^{100}GI^{101} ^{20}GV^{21} ^{17}GY^{18} ^{35}GY^{36} ^{68}HS^{69} ^{32}HT^{33} ^{95}IL^{96} ^{89}IM^{90} ^{55}IN^{56} ^{101}IN^{102}	^{60}WC^{61} ^{24}AE^{25} ^{106}AH^{107} ^{63}DN^{64} ^{99}EG^{100} ^{39}ET^{40} ^{25}EW^{26} ^{49}EY^{50} ^{53}FQ^{54} ^{19}GG^{20} ^{17}GH^{18} ^{20}GI^{21} ^{100}GI^{101} ^{35}GY^{36} ^{29}II^{30} ^{95}IL^{96} ^{55}IN^{56} ^{59}IW^{60} ^{98}KE^{99} ^{79}KF^{80} ^{58}KI^{59} ^{94}KI^{95} ^{93}KK^{94} ^{122}KW^{123} ^{85}LT^{86} ^{15}MN^{16} ^{64}NE^{65} ^{16}NG^{17} ^{45}NG^{46} ^{66}NL^{67} ^{44}NN^{45} ^{56}NN^{57} ^{47}NR^{48}	^{105}LAHKALCSEK^{114} ^{60}WCKDDQNPHS^{69} ^{110}LCSEKLDQWL^{119} ^{31}FHTSGYDTQA^{40} ^{33}TSGYDTQAIV^{42} ^{37}DTQAIVQNND^{46} 104WL^{105} ^{118}WL^{119} ^{60}WC^{61} ^{30}AF^{31} ^{106}AH^{107} ^{64}DQ^{65} ^{116}DQ^{117} ^{7}EV^{8} ^{25}EW^{26} ^{49}EY^{50} ^{9}FQ^{10} ^{53}FQ^{54} ^{19}GG^{20} ^{100}GI^{101} ^{20}GV^{21} ^{35}GY^{36} ^{68}HS^{69} ^{32}HT^{33} ^{95}IL^{96} ^{55}IN^{56} ^{101}IN^{102} ^{59}IW^{60} ^{79}KF^{80} ^{58}KI^{59} ^{94}KI^{95} ^{93}KK^{94} ^{98}KV^{99}	^{109}AL^{110} ^{22}SL^{23} ^{51}GL^{52} ^{105}LAHKALCSEK^{114} ^{60}WCKDDQNPHS^{69} ^{110}LCSEKLDQWL^{119} ^{31}FHTSGYDTQA^{40} ^{33}TSGYDTQAIV^{42} ^{37}DTQAIVQNND^{46} 104WL^{105} ^{118}WL^{119} ^{60}WC^{61} ^{30}AF^{31} ^{106}AH^{107} ^{64}DQ^{65} ^{116}DQ^{117} ^{7}EV^{8} ^{25}EW^{26} ^{49}EY^{50} ^{9}FQ^{10} ^{53}FQ^{54} ^{19}GG^{20} ^{100}GI^{101} ^{20}GV^{21} ^{35}GY^{36} ^{68}HS^{69} ^{32}HT^{33} ^{95}IL^{96} ^{55}IN^{56} ^{101}IN^{102} ^{59}IW^{60} ^{79}KF^{80} ^{58}KI^{59}

	^{59}IW^{60} ^{79}KF^{80} ^{16}KG^{17} ^{58}KI^{59} ^{94}KI^{95} ^{93}KK^{94} ^{98}KV^{99} ^{3}LT^{4} ^{85}LT^{86} ^{45}ND^{46} ^{44}NN^{45} ^{56}NN^{57} ^{102}NY^{103} ^{67}PH^{68} ^{39}QA^{40} ^{54}QI^{55} ^{2}QL^{3} ^{43}QN^{44} ^{65}QN^{66} ^{117}QW^{118} ^{86}TD^{87} ^{48}TE^{49} ^{30}TF^{31} ^{4}TK^{5} ^{38}TQ^{39} ^{33}TS^{34} ^{29}TT^{30} ^{8}VF^{9} ^{99}VG^{100} ^{92}VK^{93} ^{42}VQ^{43} ^{21}VS^{22} ^{36}YD^{37}	^{2}QF^{3} ^{54}QI^{55} ^{68}QS^{69} ^{117}QW^{118} ^{70}RN^{71} ^{86}TD^{87} ^{38}TE^{39} ^{4}TK^{5} ^{22}TL^{23} ^{40}TV^{41} ^{42}VS^{43} ^{36}YD^{37} ^{50}YG^{51} ^{103}YW^{104}	^{3}LT^{4} ^{85}LT^{86} ^{45}ND^{46} ^{44}NN^{45} ^{56}NN^{57} ^{102}NY^{103} ^{67}PH^{68} ^{39}QA^{40} ^{54}QI^{55} ^{2}QL^{3} ^{43}QN^{44} ^{65}QN^{66} ^{117}QW^{118} ^{70}RN^{71} ^{86}TD^{87} ^{48}TE^{49} ^{4}TK^{5} ^{38}TQ^{39} ^{33}TS^{34} ^{8}VF^{9} ^{99}VG^{100} ^{42}VQ^{43} ^{21}VS^{22} ^{36}YD^{37} ^{18}YG^{19} ^{50}YG^{51} ^{103}YW^{104}	^{94}KI^{95} ^{93}KK^{94} ^{98}KV^{99} ^{3}LT^{4} ^{85}LT^{86} ^{45}ND^{46} ^{44}NN^{45} ^{56}NN^{57} ^{102}NY^{103} ^{67}PH^{68} ^{39}QA^{40} ^{54}QI^{55} ^{2}QL^{3} ^{43}QN^{44} ^{65}QN^{66} ^{117}QW^{118} ^{70}RN^{71} ^{86}TD^{87} ^{48}TE^{49} ^{4}TK^{5} ^{38}TQ^{39} ^{33}TS^{34} ^{8}VF^{9} ^{99}VG^{100} ^{42}VQ^{43} ^{21}VS^{22} ^{36}YD^{37} ^{18}YG^{19} ^{50}YG^{51} ^{103}YW^{104}

	^{18}YG^{19} ^{50}YG^{51} ^{103}YW^{104}			
β-lactoglobulin	ID: P02754 ^{15}VA^{16} ^{24}MA^{25} ^{138}KA^{139} ^{141}KA^{142} ^{22}LA^{23} ^{117}LA^{118} ^{37}AP^{38} ^{79}PA^{80} ^{143}LP^{144} ^{31}LL^{32} ^{57}LL^{58} ^{103}LL^{104} ^{78}IPA^{80} ^{46}LKPTOEGDL^{54} ^{46}LKPTPEGDLEIL^{57} ^{78}IP^{79} ^{49}TP^{50} ^{125}TP^{126} ^{47}KP^{48} ^{15}VAGTWY^{20} ^{72}IA^{73} ^{112}EP^{113} ^{152}NP^{153} ^{74}EK^{75} ^{134}EK^{135} ^{86}AL^{87} ^{132}AL^{133} ^{139}AL^{140} ^{142}AL^{143}	--------	ID: P02756 ^{20}YS^{21} ^{15}VAGTWY^{20} ^{19}WY^{20} ^{130}KE^{131} ^{15}VA^{16} ^{24}MA^{25} ^{138}KA^{139} ^{141}KA^{142} ^{22}LA^{23} ^{117}LA^{118} ^{149}LA^{150} ^{37}AP^{38} ^{79}PA^{80} ^{143}LP^{144} ^{31}LL^{32} ^{57}LL^{58} ^{103}LL^{104} ^{78}IPA^{80} ^{78}IP^{79} ^{49}TP^{50} ^{125}TP^{126} ^{47}KP^{48} ^{72}IA^{73} ^{112}EP^{113} ^{152}NP^{153} ^{74}EK^{75} ^{134}EK^{135} ^{86}AL^{87} ^{132}AL^{133}	ID: P67976 ^{129}DN^{130} ^{20}HS^{21} ^{130}NE^{131} ^{19}WH^{20} ^{15}VA^{16} ^{24}MA^{25} ^{138}KA^{139} ^{141}KA^{142} ^{22}LA^{23} ^{117}LA^{118} ^{149}LA^{150} ^{37}AP^{38} ^{79}PA^{80} ^{143}LP^{144} ^{31}LL^{32} ^{57}LL^{58} ^{103}LL^{104} ^{78}IPA^{80} ^{78}IP^{79} ^{49}TP^{50} ^{125}TP^{126} ^{47}KP^{48} ^{72}IA^{73} ^{112}EP^{113} ^{152}NP^{153} ^{74}EK^{75} ^{134}EK^{135} ^{86}AL^{87} ^{132}AL^{133}

	^{21}SL^{22} ^{30}SL^{31} ^{116}SL^{117} ^{9}GL^{10} ^{123}VR^{124} ^{125}TPEVDDEALEK^{135} ^{78}IPAVF^{82} ^{78}IPAVFK^{83} ^{92}VLVLDTDYK^{100} ^{78}IPAVFKIDAL^{87} ^{25}AA^{26} ^{38}PL^{39} ^{19}WY^{20} ^{61}WE^{62} ^{73}AE^{74} ^{111}AE^{112} ^{16}AG^{17} ^{26}AS^{27} ^{80}AV^{81} ^{51}EG^{52} ^{55}EI^{56} ^{127}EV^{128} ^{151}FN^{152} ^{64}GE^{65} ^{146}HI^{147} ^{161}HI^{162} ^{71}II^{72} ^{56}IL^{57} ^{12}IQ^{13} ^{147}IR^{148} ^{135}KF^{136} ^{8}KG^{9} ^{70}KI^{71}		^{139}AL^{140} ^{142}AL^{143} ^{21}SL^{22} ^{30}SL^{31} ^{116}SL^{117} ^{9}GL^{10} ^{123}VR^{124} ^{78}IPAVF^{82} ^{78}IPAVFK^{83} ^{92}VLVLDTDYK^{100} ^{78}IPAVFKIDAL ^{87} ^{25}AA^{26} ^{38}PL^{39} ^{61}WE^{62} ^{73}AE^{74} ^{111}AE^{112} ^{150}AF^{151} ^{16}AG^{17} ^{26}AS^{27} ^{80}AV^{81} ^{51}EG^{52} ^{157}EG^{158} ^{55}EI^{56} ^{127}EV^{128} ^{151}FN^{152} ^{64}GE^{65} ^{146}HI^{147} ^{161}HV^{162} ^{1}II^{2} ^{71}II^{72} ^{56}IL^{57} ^{12}IQ^{13} ^{147}IR^{148}	^{139}AL^{140} ^{142}AL^{143} ^{21}SL^{22} ^{30}SL^{31} ^{116}SL^{117} ^{9}GL^{10} ^{123}VR^{124} ^{78}IPAVF^{82} ^{78}IPAVFK^{83} ^{92}VLVLDTDYK^{100} ^{78}IPAVFKIDAL ^{87} ^{25}AA^{26} ^{38}PL^{39} ^{61}WE^{62} ^{73}AE^{74} ^{111}AE^{112} ^{150}AF^{151} ^{16}AG^{17} ^{26}AS^{27} ^{80}AV^{81} ^{51}EG^{52} ^{157}EG^{158} ^{55}EI^{56} ^{127}EV^{128} ^{151}FN^{152} ^{64}GE^{65} ^{146}HI^{147} ^{161}HV^{162} ^{1}II^{2} ^{71}II^{72} ^{56}IL^{57} ^{12}IQ^{13} ^{147}IR^{148}

	^{77}KI^{78} ^{83}KI^{84} ^{69}KK^{70} ^{100}KK^{101} ^{75}KT^{76} ^{14}KV^{15} ^{91}KV^{92} ^{60}KW^{61} ^{101}KY^{102} ^{1}LI^{2} ^{87}LN^{88} ^{93}LV^{94} ^{122}LV^{123} ^{107}ME^{108} ^{145}MH^{146} ^{7}MK^{8} ^{88}NE^{89} ^{63}NG^{64} ^{144}PM^{145} ^{48}PT^{49} ^{153}PT^{154} ^{155}QL^{156} ^{35}QS^{36} ^{115}QS^{116} ^{5}QT^{6} ^{148}RL^{149} ^{150}SF^{151} ^{97}TD^{98} ^{76}TK^{77} ^{6}TM^{7} ^{4}TQ^{5} ^{154}TQ^{155} ^{18}TW^{19}		^{135}KF^{136} ^{8}KG^{9} ^{70}KI^{71} ^{77}KI^{78} ^{83}KI^{84} ^{69}KK^{70} ^{100}KK^{101} ^{75}KT^{76} ^{14}KV^{15} ^{91}KV^{92} ^{60}KW^{61} ^{101}KY^{102} ^{87}LN^{88} ^{93}LV^{94} ^{122}LV^{123} ^{107}ME^{108} ^{145}MH^{146} ^{7}MK^{8} ^{88}NE^{89} ^{63}NG^{64} ^{53}NL^{54} ^{144}PM^{145} ^{48}PT^{49} ^{153}PT^{154} ^{155}QL^{156} ^{35}QS^{36} ^{115}QS^{116} ^{5}QT^{6} ^{148}RL^{149} ^{97}TD^{98} ^{76}TK^{77} ^{6}TM^{7} ^{4}TQ^{5}	^{135}KF^{136} ^{8}KG^{9} ^{70}KI^{71} ^{77}KI^{78}, ^{83}KI^{84} ^{69}KK^{70} ^{100}KK^{101} ^{75}KT^{76} ^{14}KV^{15} ^{91}KV^{92} ^{60}KW^{61} ^{101}KY^{102} ^{87}LN^{88} ^{93}LV^{94} ^{122}LV^{123} ^{107}ME^{108} ^{145}MH^{146} ^{7}MK^{8} ^{88}NE^{89} ^{63}NG^{64} ^{53}NL^{54} ^{144}PM^{145} ^{48}PT^{49} ^{153}PT^{154} ^{155}QL^{156} ^{35}QS^{36} ^{115}QS^{116} ^{5}QT^{6} ^{148}RL^{149} ^{97}TD^{98} ^{76}TK^{77} ^{6}TM^{7} ^{4}TQ^{5}

	^{128}VD^{129} ^{43}VE^{44} ^{81}VF^{82} ^{92}VL^{93} ^{94}VL^{95} ^{3}VT^{4} ^{41}VY^{42} ^{99}YK^{100} ^{102}YL^{103} ^{20}YS^{21} ^{42}YV^{43}		^{154}TQ^{155} ^{18}TW^{19} ^{128}VD^{129} ^{43}VE^{44} ^{81}VF^{82} ^{92}VL^{93} ^{94}VL^{95} ^{3}VT^{4} ^{41}VY^{42} ^{99}YK^{100} ^{102}YL^{103} ^{42}YV^{43}	^{154}TQ^{155} ^{18}TW^{19} ^{128}VD^{129} ^{43}VE^{44} ^{81}VF^{82} ^{92}VL^{93} ^{94}VL^{95} ^{3}VT^{4} ^{41}VY^{42} ^{99}YK^{100} ^{102}YL^{103} ^{42}YV^{43}
Lactoferrin	ID: D0VAV0 ^{382}VL^{383} ^{384}VL^{385} ^{410}VL^{411} ^{426}VL^{427} ^{610}VL^{611} ^{383}LV^{384} ^{407}LV^{408} ^{381}IV^{382} ^{474}IV^{475} ^{131}IL^{132} ^{266}IL^{267} ^{473}IL^{474} ^{126}II^{127} ^{229}LL^{230} ^{270}LL^{271} ^{298}LL^{299} ^{571}LL^{572} ^{611}LL^{612}	ID: Q9TUM0 ^{384}VL^{385} ^{410}VL^{411} ^{548}VL^{549} ^{610}VL^{611} ^{63}LV^{64} ^{271}LV^{272} ^{383}LV^{384} ^{407}LV^{408} ^{266}LI^{267} ^{131}LL^{132} ^{229}LL^{230} ^{270}LL^{271} ^{298}LL^{299} ^{307}LL^{308} ^{473}LL^{474} ^{571}LL^{572} ^{611}LL^{612} ^{639}LL^{640}	ID: Q29477 ^{384}VL^{385} ^{426}VL^{427} ^{610}VL^{611} ^{383}LV^{384} ^{407}LV^{408} ^{131}IL^{132} ^{266}IL^{267} ^{473}IL^{474} ^{229}LL^{230} ^{270}LL^{271} ^{298}LL^{299} ^{571}LL^{572} ^{611}LL^{612} ^{639}LL^{640} ^{680}LL^{681} ^{346}VW^{347} ^{548}VW^{549} ^{351}GP^{352}	ID: D3G9G3 ^{384}VL^{385} ^{426}VL^{427} ^{610}VL^{611} ^{383}LV^{384} ^{407}LV^{408} ^{131}IL^{132} ^{266}IL^{267} ^{473}IL^{474} ^{229}LL^{230} ^{270}LL^{271} ^{298}LL^{299} ^{571}LL^{572} ^{611}LL^{612} ^{639}LL^{640} ^{680}LL^{681} ^{346}VW^{347} ^{548}VW^{549} ^{351}GP^{352}

	^{639}LL^{640}	^{680}LL^{681}	^{77}VA^{78}	^{77}VA^{78}
	^{680}LL^{681}	^{346}VW^{347}	^{95}VA^{96}	^{95}VA^{96}
	^{346}VW^{347}	^{31}GP^{32}	^{149}VA^{150}	^{149}VA^{150}
	^{548}VW^{549}	^{140}GP^{141}	^{206}VA^{207}	^{206}VA^{207}
	^{351}GP^{352}	^{662}GP^{663}	^{256}VA^{257}	^{256}VA^{257}
	^{292}PP^{293}	^{141}PP^{142}	^{436}VA^{437}	^{436}VA^{437}
	^{77}VA^{78}	^{95}VA^{96}	^{540}VA^{541}	^{540}VA^{541}
	^{95}VA^{96}	^{149}VA^{150}	^{591}VA^{592}	^{591}VA^{592}
	^{149}VA^{150}	^{206}VA^{207}	^{411}MA^{412}	^{411}MA^{412}
	^{206}VA^{207}	^{256}VA^{257}	^{164}KA^{165}	^{164}KA^{165}
	^{256}VA^{257}	^{436}VA^{437}	^{221}KA^{222}	^{221}KA^{222}
	^{436}VA^{437}	^{540}VA^{541}	^{273}KA^{274}	^{273}KA^{274}
	^{540}VA^{541}	^{591}VA^{592}	^{339}KA^{340}	^{339}KA^{340}
	^{591}VA^{592}	^{604}VA^{605}	^{441}KA^{442}	^{441}KA^{442}
	^{53}KA^{54}	^{53}KA^{54}	^{247}LA^{248}	^{247}LA^{248}
	^{221}KA^{222}	^{147}KA^{148}	^{434}LA^{435}	^{434}LA^{435}
	^{273}KA^{274}	^{221}KA^{222}	^{533}LA^{534}	^{533}LA^{534}
	^{339}KA^{340}	^{273}KA^{274}	^{589}LA^{590}	^{589}LA^{590}
	^{441}KA^{442}	^{441}KA^{442}	^{648}LA^{649}	^{648}LA^{649}
	^{247}LA^{248}	^{247}LA^{248}	^{1}AP^{2}	^{1}AP^{2}
	^{411}LA^{412}	^{411}LA^{412}	^{31}AP^{32}	^{31}AP^{32}
	^{434}LA^{435}	^{434}LA^{435}	^{237}AP^{238}	^{237}AP^{238}
	^{533}LA^{534}	^{533}LA^{534}	^{492}AP^{493}	^{492}AP^{493}
	^{589}LA^{590}	^{589}LA^{590}	^{592}AP^{593}	^{592}AP^{593}
	^{648}LA^{649}	^{648}LA^{649}	^{13}LP^{14}	^{218}LP^{219}
	^{41}FA^{42}	^{492}AP^{493}	^{218}LP^{219}	^{158}VP^{159}
	^{1}AP^{2}	^{592}AP^{593}	^{158}VP^{159}	^{250}VP^{251}
	^{31}AP^{32}	^{13}PA^{14}	^{250}VP^{251}	^{408}VP^{409}
	^{237}AP^{238}	^{219}PA^{220}	^{408}VP^{409}	^{516}VP^{517}
	^{492}AP^{493}	^{292}PA^{293}	^{516}VP^{517}	^{229}VP^{230}
	^{592}AP^{593}	^{218}LP^{219}	^{229}VP^{230}	^{270}VP^{271}
	^{218}LP^{219}	^{158}VP^{159}	^{270}VP^{271}	^{298}VP^{299}
	^{158}VP^{159}	^{250}VP^{251}	^{298}VP^{299}	^{571}VP^{572}

	^{250}VP^{251}	^{408}VP^{409}	^{571}VP^{572}	^{611}VP^{612}
	^{408}VP^{409}	^{516}VP^{517}	^{611}VP^{612}	^{639}VP^{640}
	^{516}VP^{517}	^{131}LL^{132}	^{639}VP^{640}	^{680}VP^{681}
	^{229}LL^{230}	^{229}LL^{230}	^{680}VP^{681}	^{97}VV^{98}
	^{270}LL^{271}	^{270}LL^{271}	^{97}VV^{98}	^{255}VV^{256}
	^{298}LL^{299}	^{298}LL^{299}	^{255}VV^{256}	^{345}VV^{346}
	^{571}LL^{572}	^{307}LL^{308}	^{345}VV^{346}	^{438}VV^{439}
	^{611}LL^{612}	^{473}LL^{474}	^{438}VV^{439}	^{597}VV^{598}
	^{639}LL^{640}	^{571}LL^{572}	^{597}VV^{598}	^{253}HA^{254}
	^{680}LL^{681}	^{611}LL^{612}	^{253}HA^{254}	^{595}HA^{596}
	^{97}VV^{98}	^{639}LL^{640}	^{595}HA^{596}	^{492}APG^{494}
	^{255}VV^{256}	^{680}LL^{681}	^{492}APG^{494}	^{127}IP^{128}
	^{345}VV^{346}	^{255}VV^{256}	^{127}IP^{128}	^{310}IP^{311}
	^{438}VV^{439}	^{345}VV^{346}	^{310}IP^{311}	^{469}IP^{470}
	^{597}VV^{598}	^{369}VV^{370}	^{469}IP^{470}	^{87}SP^{88}
	^{253}HA^{254}	^{438}VV^{439}	^{87}SP^{88}	^{291}SP^{292}
	^{595}HA^{596}	^{597}VV^{598}	^{291}SP^{292}	^{678}SP^{679}
	^{492}APG^{494}	^{253}HA^{254}	^{678}SP^{679}	^{75}RP^{76}
	^{127}IP^{128}	^{595}HA^{596}	^{75}RP^{76}	^{133}RP^{134}
	^{310}IP^{311}	^{492}APG^{494}	^{133}RP^{134}	^{428}RP^{429}
	^{469}IP^{470}	^{127}IP^{128}	^{428}RP^{429}	^{654}RP^{655}
	^{87}SP^{88}	^{469}IP^{470}	^{654}RP^{655}	^{579}KP^{580}
	^{291}SP^{292}	^{12}SP^{13}	^{579}KP^{580}	^{166}YP^{167}
	^{678}SP^{679}	^{291}SP^{292}	^{166}YP^{167}	^{30}GA^{31}
	^{75}RP^{76}	^{417}SP^{418}	^{30}GA^{31}	^{147}GA^{148}
	^{133}RP^{134}	^{678}SP^{679}	^{147}GA^{148}	^{194}GA^{195}
	^{428}RP^{429}	^{75}RP^{76}	^{194}GA^{195}	^{202}GA^{203}
	^{654}RP^{655}	^{133}RP^{134}	^{202}GA^{203}	^{494}GA^{495}
	^{579}KP^{580}	^{428}RP^{429}	^{494}GA^{495}	^{528}GA^{529}
	^{166}YP^{167}	^{237}KP^{238}	^{528}GA^{529}	^{49}IA^{50}
	^{30}GA^{31}	^{282}KP^{283}	^{49}IA^{50}	^{381}IA^{382}
	^{147}GA^{148}	^{579}KP^{580}	^{381}IA^{382}	^{474I}A^{475}
	^{194}GA^{195}	^{166}YP^{167}	^{474I}A^{475}	^{669}IA^{670}

	^{202}GA^{203} ^{494}GA^{495} ^{528}GA^{529} ^{49}IA^{50} ^{669}IA^{670} ^{39}RA^{40} ^{47}RA^{48} ^{236}RA^{237} ^{603}RA^{604} ^{560}WA^{561} ^{143}EP^{144} ^{187}EP^{188} ^{334}TA^{335} ^{373}TA^{374} ^{401}TA^{402} ^{459}TA^{460} ^{464}TA^{465} ^{557}TA^{558} ^{667}TA^{668} ^{13}QP^{14} ^{307}FL^{308} ^{686}FL^{687} ^{246}HL^{247} ^{588}HL^{589} ^{51}EK^{52} ^{220}EK^{221} ^{276}EK^{277} ^{521}EK^{522} ^{42}AL^{43} ^{304}AL^{305} ^{317}AL^{318} ^{391}AL^{392} ^{503}AL^{504}	^{194}GA^{195} ^{202}GA^{203} ^{528}GA^{529} ^{77}IA^{78} ^{97}IA^{98} ^{381}IA^{382} ^{401}IA^{402} ^{669}IA^{670} ^{342}RA^{343} ^{560}WA^{561} ^{143}EP^{144} ^{187}EP^{188} ^{87}NP^{88} ^{326}TA^{327} ^{334}TA^{335} ^{373}TA^{374} ^{459}TA^{460} ^{464}TA^{465} ^{667}TA^{668} ^{135}FL^{136} ^{486}FL^{487} ^{686}FL^{687} ^{246}HL^{247} ^{588}HL^{589} ^{606}HL^{607} ^{52}EK^{53} ^{276}EK^{277} ^{304}AL^{305} ^{382}AL^{383} ^{391}AL^{392} ^{503}AL^{504} ^{217}SL^{218} ^{393}SL^{394}	^{669}IA^{670} ^{47}RA^{48} ^{236}RA^{237} ^{603}RA^{604} ^{560}WA^{561} ^{143}EP^{144} ^{187}EP^{188} ^{326}TA^{327} ^{334}TA^{335} ^{373}TA^{374} ^{401}TA^{402} ^{459}TA^{460} ^{464}TA^{465} ^{667}TA^{668} ^{135}FL^{136} ^{686}FL^{687} ^{246}HL^{247} ^{85}EK^{86} ^{220}EK^{221} ^{276}EK^{I277} ^{521}EK^{522} ^{658}EK^{659} ^{42}AL^{43} ^{304}AL^{305} ^{317}AL^{318} ^{327}AL^{328} ^{382}AL^{383} ^{391}AL^{392} ^{503}AL^{504} ^{616}AL^{617} ^{12}SL^{13} ^{393}SL^{394} ^{422}SL^{423}	^{47}RA^{48} ^{236}RA^{237} ^{603}RA^{604} ^{560}WA^{561} ^{143}EP^{144} ^{187}EP^{188} ^{326}TA^{327} ^{334}TA^{335} ^{401}TA^{402} ^{459}TA^{460} ^{464}TA^{465} ^{667}TA^{668} ^{135}FL^{136} ^{686}FL^{687} ^{246}HL^{247} ^{85}EK^{86} ^{220}EK^{221} ^{276}EK^{I277} ^{521}EK^{522} ^{658}EK^{659} ^{42}AL^{43} ^{304}AL^{305} ^{317}AL^{318} ^{327}AL^{328} ^{382}AL^{383} ^{391}AL^{392} ^{503}AL^{504} ^{616}AL^{617} ^{393}SL^{394} ^{422}SL^{423} ^{450}SL^{451} ^{500}SL^{501} ^{68}GL^{69}

	^{616}AL^{617}	^{450}SL^{451}	^{450}SL^{451}	^{118}GL^{119}
	^{141}SL^{142}	^{62}GL^{63}	^{500}SL^{501}	^{406}GL^{407}
	^{422}SL^{423}	^{68}GL^{69}	^{68}GL^{69}	^{445}GL^{446}
	^{450}SL^{451}	^{111}GL^{112}	^{118}GL^{119}	^{472}GL^{473}
	^{118}GL^{119}	^{118}GL^{119}	^{406}GL^{407}	^{511}GL^{512}
	^{406}GL^{407}	^{130}GL^{131}	^{445}GL^{446}	^{6}VR^{7}
	^{445}GL^{446}	^{306}GL^{307}	^{472}GL^{473}	^{308}VR^{309}
	^{472}GL^{473}	^{317}GL^{318}	^{511}GL^{512}	^{78}AA^{79}
	^{511}GL^{512}	^{330}GL^{331}	^{6}VR^{7}	^{604}AA^{605}
	^{6}VR^{7}	^{406}GL^{407}	^{308}VR^{309}	^{144}PL^{145}
	^{37}VR^{38}	^{422}GL^{423}	^{78}AA^{79}	^{679}PL^{680}
	^{24}WRM^{26}	^{472}GL^{473}	^{604}AA^{605}	^{22}WQ^{23}
	^{78}AA^{79}	^{6}VR^{7}	^{144}PL^{145}	^{125}WN^{126}
	^{604}AA^{605}	^{29}VR^{30}	^{679}PL^{680}	^{448}WN^{449}
	^{144}PL^{145}	^{439}VR^{440}	^{22}WQ^{23}	^{467}WN^{468}
	^{679}PL^{680}	^{78}AA^{79}	^{125}WN^{126}	^{8}WC^{9}
	^{292}PPG^{294}	^{335}AA^{336}	^{448}WN^{449}	^{347}WC^{348}
	^{24}WR^{25}	^{144}PL^{145}	^{467}WN^{468}	^{138}WT^{139}
	^{268}WK^{269}	^{679}PL^{680}	^{8}WC^{9}	^{361}WS^{362}
	^{22}WQ^{23}	^{268}WK^{269}	^{347}WC^{348}	^{268}WE^{269}
	^{125}WI^{126}	^{22}WQ^{23}	^{138}WT^{139}	^{549}WE^{550}
	^{448}WN^{449}	^{125}WN^{126}	^{16}WS^{17}]	^{400}YT^{401}
	^{467}WN^{468}	^{448}WN^{449}	^{361}WS^{362}	^{526}YT^{527}
	^{8}WC^{9}	^{467}WN^{468}	^{268}WE^{269}	^{54}AD^{55}
	^{347}WC^{348}	^{8}WC^{9}	^{549}WE^{550}	^{222}AD^{223}
	^{138}WT^{139}	^{347}WC^{348}	^{400}YT^{401}	^{389}AD^{390}
	^{361}WS^{362}	^{138}WT^{139}	^{526}YT^{527}	^{495}AD^{496}
	^{549}WE^{550}	^{361}WS^{362}	^{54}AD^{55}	^{558}AD^{559}
	^{16}WF^{17}	^{526}YT^{527}	^{222}AD^{223}	^{79}AE^{80}
	^{342}YT^{343}	^{54}AD^{55}	^{389}AD^{390}	^{142}AE^{143}
	^{400}YT^{401}	^{222}AD^{223}	^{495}AD^{496}	^{335}AE^{336}
	^{526}YT^{527}	^{389}AD^{390}	^{558}AD^{559}	^{412}AE^{413}
	^{54}AD^{55}	^{14}AE^{15}	^{79}AE^{80}	^{534}AE^{535}

	^{222}AD^{223} ^{389}AD^{390} ^{495}AD^{496} ^{558}AD^{559} ^{50}AE^{51} ^{79}AE^{80} ^{335}AE^{336} ^{412}AE^{413} ^{534}AE^{535} ^{40}AF^{41} ^{195}AF^{196} ^{207}AF^{208} ^{241}AF^{242} ^{482}AF^{483} ^{529}AF^{530} ^{541}AF^{542} ^{685}AF^{686} ^{67}AG^{68} ^{123}AG^{124} ^{203}AG^{204} ^{402}AG^{403} ^{465}AG^{466} ^{506}AG^{507} ^{605}AH^{606} ^{155}AS^{156} ^{374}AS^{375} ^{372}AT^{373} ^{56}AV^{57} ^{94}AV^{95} ^{96}AV^{97} ^{148}AV^{149} ^{254}AV^{255} ^{349}AV^{350}	^{79}AE^{80}, ^{336}AE^{337} ^{412}AE^{413} ^{534}AE^{535} ^{584}AE^{585} ^{195}AF^{196}, ^{207}AF^{208} ^{241}AF^{242} ^{529}AF^{530} ^{541}AF^{542} ^{685}AF^{686} ^{67}AG^{68} ^{123}AG^{124} ^{174}AG^{175}, ^{203}AG^{204} ^{293}AG^{294} ^{402}AG^{403} ^{465}AG^{466} ^{506}AG^{507} ^{605}AH^{606} ^{616}AH^{617} ^{1}AS^{2} ^{155}AS^{156} ^{374}AS^{375} ^{372}AT^{373} ^{56}AV^{57} ^{94}AV^{95} ^{148}AV^{149} ^{254}AV^{255} ^{349}AV^{350} ^{435}AV^{436} ^{437}AV^{438}, ^{460}AV^{461}	^{142}AE^{143} ^{335}AE^{336} ^{412}AE^{413} ^{534}AE^{535} ^{195}AF^{196} ^{207} AF^{208} ^{241}AF^{242} ^{482}AF^{483} ^{529}AF^{530} ^{541}AF^{542} ^{685}AF^{686} ^{50}AG^{51} ^{67}AG^{68} ^{123}AG^{124} ^{203}AG^{204} ^{402}AG^{403} ^{465}AG^{466} ^{506}AG^{507} ^{605}AH^{606} ^{155}AS^{156} ^{374}AS^{375} ^{372}AT^{373} ^{56}AV^{57} ^{94}AV^{95} ^{96}AV^{97} ^{148}AV^{149} ^{254}AV^{255} ^{349}AV^{350} ^{435}AV^{436} ^{437}AV^{438} ^{460}AV^{461} ^{590}AV^{591} ^{596}AV^{597}	^{195}AF^{196} ^{207} AF^{208} ^{241}AF^{242} ^{482}AF^{483} ^{529}AF^{530} ^{541}AF^{542} ^{685}AF^{686} ^{50}AG^{51} ^{67}AG^{68} ^{123}AG^{124} ^{203}AG^{204} ^{402}AG^{403} ^{465}AG^{466} ^{506}AG^{507} ^{605}AH^{606} 155AS156 ^{374}AS^{375} ^{56}AV^{57} ^{94}AV^{95} ^{96}AV^{97} ^{148}AV^{149} ^{254}AV^{255} ^{349}AV^{350} ^{435}AV^{436} ^{437}AV^{438} ^{460}AV^{461} ^{590}AV^{591} ^{596}AV^{597} ^{165}AY^{166} ^{643}DN^{644} ^{70}DP^{71} ^{496}DP^{497} ^{107}DQ^{108}

	^{435}AV^{436}	^{590}AV^{591}	^{165}AY^{166}	^{225}DQ^{226}
	^{437}AV^{438}	^{596}AV^{597}	^{643}DN^{644}	^{509}DQ^{510}
	^{460}AV^{461}	^{550}DN^{551}	^{70}DP^{71}	^{627}DQ^{628}
	[^{590}AV^{591}	^{643}DN^{644}	^{496}DP^{497}	^{223}DR^{224}
	^{596}AV^{597}	^{70}DP^{71}	^{107}DQ^{108}	^{462}DR^{463}
	^{165}AY^{166}	^{496}DP^{497}	^{225}DQ^{226}	^{602}DR^{603}
	^{643}DN^{644}	^{225}DQ^{226}	^{509}DQ^{510}	^{431}EG^{432}
	^{70}DP^{71}	^{223}DR^{224}	^{627}DQ^{628}	^{444}EG^{445}
	^{496}DP^{497}	^{462}DR^{463}	^{223}DR^{224}	[^{80}EI^{81}
	^{107}DQ^{108}	^{510}EG^{511}	^{462}DR^{463}	^{140}ES^{141}
	^{225}DQ^{226}	^{15}ES^{16}	^{602}DR^{603}	^{555}ES^{556}
	^{509}DQ^{510}	^{216}ES^{217}	^{293}EG^{294}	^{211}ET^{212}
	^{162}DR^{163}	^{413}ES^{414}	^{431}EG^{432}	^{333}ET^{334}
	^{223}DR^{224}	^{419}ES^{420}	^{444}EG^{445}	^{635}ET^{636}
	^{462}DR^{463}	^{585}ES^{586}	[^{80}EI^{81}	^{664}EY^{665}
	^{602}DR^{603}	^{333}ET^{334}	^{140}ES^{141}	^{641}FN^{642}
	^{176}EG^{177}	^{80}EV^{81}	^{555}ES^{556}	^{104}FQ^{105}
	^{431}EG^{432}	^{337}EV^{338}	^{211}ET^{212}	^{286}FQ^{287}
	^{444}EG^{445}	^{360}EW^{361}	^{333}ET^{334}	^{530}FR^{531}
	^{80}EI^{81}	^{165}EY^{166}	^{635}ET^{636}	^{569}FR^{570}
	^{86}ES^{87}	^{659}EY^{660}	^{15}EW^{16}	^{177}GE^{178}
	^{140}ES^{141}	^{641}FN^{642}	^{664}EY^{665}	^{387}GE^{388}
	^{555}ES^{556}	^{104}FQ^{105}	^{641}FN^{642}	^{554}GE^{555}
	^{211}ET^{212}	^{242}FQ^{243}	^{104}FQ^{105}	^{306}GF^{307}
	^{333}ET^{334}	^{286}FQ^{287}	^{286}FQ^{287}	^{396}GG^{397}
	^{635}ET^{636}	^{632}FQ^{633}	^{530}FR^{531}	^{652}GG^{653}
	^{337}EV^{338}	^{530}FR^{531}	^{569}FR^{570}	^{130}GI^{131}
	^{15}EW^{16}	^{177}GE^{178}	^{177}GE^{178}	^{175}GV^{176}
	^{659}EY^{660}	^{387}GE^{388}	^{387}GE^{388}	^{124}GW^{125}
	^{664}EY^{665}	^{285}GF^{286}	^{554}GE^{555}	^{466}GW^{467}
	^{641}FN^{642}	^{61}GG^{62}	^{306}GF^{307}	^{191}GY^{192}
	^{104}FQ^{105}	^{396}GG^{397}	^{396}GG^{397}	^{397}GY^{398}
	^{286}FQ^{287}	^{124}GW^{125}	^{652}GG^{653}	^{432}GY^{433}

	^{530}FR^{531}	^{466}GW^{467}	^{130}GI^{131}	^{525}GY^{526}
	^{569}FR^{570}	^{191}GY^{192}	^{175}GV^{176}	^{458}HT^{459}
	^{175}GE^{176}	^{397}GY^{398}	^{124}GW^{125}	^{606}HV^{607}
	^{177}GE^{178}	^{432}GY^{433},	^{466}GW^{467}	^{91}HY^{92}
	^{387}GE^{388}	^{525}GY^{526}	^{191}GY^{192}	^{131}IL^{132}
	^{554}GE^{555}	^{617}HF^{618}	^{397}GY^{398}	^{46}IR^{47}
	^{306}GF^{307}	^{427}HR^{428}	^{432}GY^{433}	^{267}IW^{268}
	^{61}GG^{62}	^{116}HT^{117}	^{525}GY^{526}	^{210}KE^{211}
	^{396}GG^{397}	^{458}HT^{459}	^{458}HT^{459}	^{243}KE^{244}
	^{652}GG^{653}	^{91}HY^{92}	^{606}HV^{607}	^{263}KE^{264}
	^{130}GI^{131}	^{46}IQ^{47}	^{91}HY^{92}	^{520}KE^{521}
	^{124}GW^{125}	^{328}IR^{329}	^{131}IL^{132}	^{277}KF^{278}
	^{466}GW^{467}	^{267}IW^{268}	^{37}IR^{38}	^{100}KG^{101}
	^{191}GY^{192}	^{164}KE^{165}	^{46}IR^{47}	^{174}KG^{175}
	^{397}GY^{398}	^{263}KE^{264}	^{267}IW^{268}	^{386}KG^{387}
	^{432}GY^{433}	^{151}KF^{152}	^{210}KE^{211}	^{452}KG^{453}
	^{525}GY^{526}	^{277}KF^{278}	^{243}KE^{244}	^{99}KK^{100}
	^{420}HS^{421}	^{628}KF^{629}	^{263}KE^{264}	^{440}KK^{441}
	^{116}HT^{117}	^{100}KG^{101}	^{520}KE^{521}	^{454}KK^{455}
	^{458}HT^{459}	^{386}KG^{387}	^{277}KF^{278}	^{673}KK^{674}
	^{606}HV^{607}	^{431}KG^{432}	^{100}KG^{101}	^{86}KS^{87}
	^{91}HY^{92}	^{313}KI^{314}	^{174}KG^{175}	^{113}KS^{114}
	^{126}II^{127}	^{445}KI^{446}	^{386}KG^{387}	^{282}KS^{283}
	^{131}IL^{132}	^{3}KK^{4}	^{452}KG^{453}	^{455}KS^{456}
	^{46}IR^{47}	^{27}KK^{28}	^{99}KK^{100}	^{498}KS^{499}
	^{267}IW^{268}	^{38}KK^{39}	^{440}KK^{441}	^{633}KS^{634}
	^{85}KE^{86}	^{99}KK^{100}	^{454}KK^{455}	^{313}KV^{314}
	^{210}KE^{211}	^{454}KK^{455}	^{673}KK^{674}	^{419}KY^{420}
	^{243}KE^{244}	^{4}KS^{5}	^{86}KS^{87}	^{522}KY^{523}
	^{263}KE^{264}	^{113}KS^{114}	^{113}KS^{114}	^{659}KY^{660}
	^{520}KE^{521}	^{455}KS^{456}	^{282}KS^{283}	^{612}LH^{613}
	^{151}KF^{152}	^{39}KT^{40}	^{416}KS^{417}	^{266}LI^{267}
	^{277}KF^{278}	^{635}KT^{636}	^{455}KS^{456}	^{473}LI^{474}

	^{628}KF^{629}	^{654}KT^{655}	^{498}KS^{499}	^{232}LN^{233}
	^{100}KG^{101}	^{28}KV^{29}	^{633}KS^{634}	^{564}LN^{565}
	^{174}KG^{175}	^{603}KV^{604}	^{313}KV^{314}	^{325}LT^{326}
	^{386}KG^{387}	^{266}LI^{267}	^{419}KY^{420}	^{446}LT^{447}
	^{419}KH^{420}	^{687}LM^{688}	^{522}KY^{523}	^{687}LT^{688}
	^{27}KK^{28}	^{106}LN^{107}	^{659}KY^{660}	^{383}LV^{384}
	^{52}KK^{53}	^{574}LN^{575}	^{612}LH^{613}	^{407}LV^{408}
	^{99}KK^{100}	^{63}LV^{64}	^{266}LI^{267}	^{117}MG^{118}
	^{356}KK^{357}	^{271}LV^{272}	^{473}LI^{474}	^{471}MG^{472}
	^{440}KK^{441}	^{383}LV^{384}	^{232}LN^{233}	^{26}MR^{27}
	^{454}KK^{455}	^{407}LV^{408}	^{564}LN^{565}	^{63}MV^{64}
	^{673}KK^{674}	^{129}MG^{130}	^{325}LT^{326}	^{53}NA^{54}
	^{113}KS^{114}	^{471}MG^{472}	^{446}LT^{447}	^{545}ND^{546}
	^{282}KS^{283}	^{26}MK^{27}	^{687}LT^{688}	^{642}ND^{643}
	^{416}KS^{417}	^{688}MR^{689}	^{383}LV^{384}	^{443}NE^{444}
	^{455}KS^{456}	^{443}ND^{444}	^{407}LV^{408}	^{103}NF^{104}
	^{498}KS^{499}	^{642}ND^{643}	^{117}MG^{118}	^{553}NG^{554}
	^{633}KS^{634}	^{508}NE^{509}	^{471}MG^{472}	^{621}NG^{622}
	^{313}KV^{314}	^{103}NF^{104}	^{26}MR^{27}	^{594}NH^{595}
	^{522}KY^{523}	^{261}NG^{262}	^{63}MV^{64}	^{168}NL^{169}
	^{612}LH^{613}	^{575}NG^{576}	^{53}NA^{54}	^{217}NL^{218}
	^{266}LI^{267}	^{621}NG^{622}	^{545}ND^{546}	^{265}NL^{266}
	^{473}LI^{474}	^{594}NH^{595}	^{642}ND^{643}	^{330}NL^{331}
	^{232}LN^{233}	^{168}NL^{169}	^{443}NE^{444}	^{563}NL^{564}
	^{392}LN^{393}	^{638}NL^{639}	^{103}NF^{104}	^{638}NL^{639}
	^{564}LN^{565}	^{86}NN^{87}	^{553}NG^{554}	^{671}NL^{672}
	^{325}LT^{326}	^{233}NN^{234}	^{621}NG^{622}	^{233}NN^{234}
	^{446}LT^{447}	^{107}NQ^{108}	^{594}NH^{595}	^{476}NQ^{477}
	^{687}LT^{688}	^{366}NQ^{367}	^{168}NL^{169}	^{414}NR^{415}
	^{383}LV^{384}	^{234}NT^{235}	^{217}NL^{218}	^{565}NR^{566}
	^{407}LV^{408}	^{477}NT^{478}	^{265}NL^{266}	^{234}NT^{235}
	^{129}MG^{130}	^{551}NT^{552}	^{330}NL^{331}	^{551}NT^{552}
	^{471}MG^{472}	^{556}NT^{557}	^{563}NL^{564}	^{644}NT^{645}

	^{26}MK^{27}	^{644}NT^{645}	^{638}NL^{639}	^{5}NV^{6}
	^{63}MV^{64}	^{536}NV^{537}	^{671}NL^{672}	^{368}NV^{369}
	^{545}ND^{546}	^{323}NY^{324}	^{233}NN^{234}	^{134}PF^{135}
	^{642}ND^{643}	^{134}PF^{135}	^{476}NQ^{477}	^{493}PG^{494}
	^{443}NE^{444}	^{493}PG^{494}	^{414}NR^{415}	^{497}PK^{498}
	^{103}NF^{104}	^{626}PG^{627}	^{565}NR^{566}	^{470}PM^{471}
	^{553}NG^{554}	^{76}PI^{77}	^{234}NT^{235}	^{167}PN^{168}
	^{621}NG^{622}	^{128}PM^{129}	^{551}NT^{552}	^{517}PN^{518}
	^{594}NH^{595}	^{470}PM^{471}	^{644}NT^{645}	^{593}PN^{594}
	^{168}NL^{169}	^{167}PN^{168}	^{5}NV^{6}	^{88}PQ^{89}
	^{217}NL^{218}	^{232}PN^{233}	^{368}NV^{369}	^{32}PS^{33}
	^{330}NL^{331}	^{517}PN^{518}	^{134}PF^{135}	^{251}PS^{252}
	^{393}NL^{394}	^{593}PN^{594}	^{493}PG^{494}	^{311}PS^{312}
	^{563}NL^{564}	^{88}PQ^{89}	^{497}PK^{498}	^{429}PT^{430}
	^{638}NL^{639}	^{663}PQ^{664}	^{470}PM^{471}	^{655}PT^{656}
	^{671}NL^{672}	^{32}PS^{33}	^{167}PN^{168}	^{76}PV^{77}
	^{233}NN^{234}	^{251}PS^{252}	^{517}PN^{518}	^{238}PV^{239}
	^{179}NQ^{180}	^{283}PS^{284}	^{593}PN^{594}	^{409}PV^{410}
	^{476}NQ^{477}	^{238}PV^{239}	^{88}PQ^{89}	^{580}PV^{581}
	^{414}NR^{415}	^{409}PV^{410}	^{32}PS^{33}	^{71}PY^{72}
	^{565}NR^{566}	^{429}PV^{430}	^{251}PS^{252}	^{188}PY^{189}
	^{551}NT^{552}	^{580}PV^{581}	^{311}PS^{312}	^{615}QA^{616}
	^{644}NT^{645}	^{71}PY^{72}	^{429}PT^{430}	^{200}QD^{201}
	^{5}NV^{6}	^{188}PY^{189}	^{655}PT^{656}	^{186}QE^{187}
	^{368}NV^{369}	^{47}QA^{48}	^{76}PV^{77}	^{275}QE^{276}
	^{293}PG^{294}	^{615}QA^{616}	^{128}PV^{129}	^{628}QF^{629}
	^{493}PG^{494}	^{200}QD^{201}	^{238}PV^{239}	^{110}QG^{111}
	^{497}PK^{498}	^{186}QE^{187}	^{409}PV^{410}	^{146}QG^{147}
	^{128}PM^{129}	^{243}QE^{244}	^{580}PV^{581}	^{510}QG^{511}
	^{470}PM^{471}	^{275}QE^{276}	^{71}PY^{72}	^{105}QL^{106}
	^{167}PN^{168}	^{359}QE^{360}	^{188}PY^{189}	^{108}QL^{109}
	^{517}PN^{518}	^{110}QG^{111}	^{615}QA^{616}	^{171}QL^{172}
	^{593}PN^{594}	^{652}QG^{653}	^{200}QD^{201}	^{287}QL^{288}

	^{88}PQ^{89}	^{105}QL^{106}	^{186}QE^{187}	^{367}QN^{368}
	^{32}PS^{33}	^{108}QL^{109}	^{275}QE^{276}	^{359}QQ^{360}
	^{251}PS^{252}	^{171}QL^{172}	^{628}QF^{629}	^{614}QQ^{615}
	^{311}PS^{312}	^{287}QL^{288}	^{110}QG^{111}	^{364}QS^{365}
	^{429}PT^{430}	^{355}QL^{356}	^{146}QG^{147}	^{489}QS^{490}
	^{655}PT^{656}	^{512}QN^{513}	^{510}QG^{511}	^{585}QS^{586}
	^{76}PV^{77}	^{415}QQ^{416}	^{105}QL^{106}	^{89}QT^{90}
	^{238}PV^{239}	^{614}QQ^{615}	^{108}QL^{109}	^{477}QT^{478}
	^{409}PV^{410}	^{364}QS^{365}	^{171}QL^{172}	^{249}QV^{250}
	^{580}PV^{581}	^{367}QS^{368}	^{287}QL^{288}	^{609}QV^{610}
	^{71}PY^{72}	^{416}QS^{417}	^{367}QN^{368}	^{21}QW^{22}
	^{134}PY^{135}	^{489}QS^{490}	^{359}QQ^{360}	^{360}QW^{361}
	^{188}PY^{189}	^{633}QS^{634}	^{614}QQ^{615}	^{226}QY^{227}
	^{164}QA^{165}	^{89}QT^{90}	^{284}QS^{285}	^{309}RI^{310}
	^{615}QA^{616}	^{344}QV^{345}	^{355}QS^{356}	^{3}RK^{4}
	^{200}QD^{201}	^{609}QV^{610}	^{364}QS^{365}	^{27}RK^{28}
	^{275}QE^{276}	^{21}QW^{22}	^{489}QS^{490}	^{272}RK^{273}
	^{110}QG^{111}	^{559}QW^{560}	^{585}QS^{586}	^{570}RL^{571}
	^{146}QG^{147}	^{226}QY^{227}	^{89}QT^{90}	^{25}RM^{26}
	^{510}QG^{511}	^{664}QY^{665}	^{477}QT^{478}	^{24}RR^{25}
	^{105}QL^{106}	^{30}RG^{31}	^{249}QV^{250}	^{38}RR^{39}
	^{108}QL^{109}	^{280}RG^{281}	^{609}QV^{610}	^{295}RR^{296}
	^{171}QL^{172}	^{329}RG^{330}	^{21}QW^{22}	^{7}RW^{8}
	^{287}QL^{288}	^{452}RG^{453}	^{360}QW^{361}	^{252}SH^{253}
	^{367}QN^{368}	^{309}RI^{310}	^{226}QY^{227}	^{33}SI^{34}
	^{359}QQ^{360}	^{600}RI^{601}	^{309}RI^{310}	^{17}SK^{18}
	^{363}QQ^{364}	^{236}RK^{237}	^{3}RK^{4}	^{312}SK^{313}
	^{614}QQ^{615}	^{440}RK^{441}	^{27}RK^{28}	^{356}SK^{357}
	^{364}QS^{365}	^{578}RK^{579}	^{272}RK^{273}	^{418}SK^{419}
	^{489}QS^{490}	^{25}RM^{26}	^{415}RK^{416}	^{519}SK^{520}
	^{585}QS^{586}	^{620}RN^{621}	^{570}RL^{571}	^{259}SK^{260}
	^{89}QT^{90}	^{24}RR^{25}	^{25}RM^{26}	^{137}SW^{138}
	^{477}QT^{478}	^{341}RR^{342}	^{24}RR^{25}	^{377}TD^{378}

	^{249}QV^{250}	^{673}RR^{674}	^{38}RR^{39}	^{84}TE^{85}
	^{609}QV^{610}	^{7}RW^{8}	^{295}RR^{296}	^{139}TE^{140}
	^{23}QW^{24}	^{252}SH^{253}	^{7}RW^{8}	^{430}TE^{431}
	^{360}QW^{361}	^{2}SK^{3}	^{285}SF^{286}	^{582}TE^{583}
	^{226}QY^{227}	^{17}SK^{18}	^{252}SH^{253}	^{645}TE^{646}
	^{309}RI^{310}	^{312}SK^{313},	^{33}SI^{34}	^{663}TE^{664}
	^{3}RK^{4}	^{475}SK^{476}	^{17}SK^{18}	^{478}TG^{479}
	^{112}RK^{113}	^{499}SK^{500}	^{312}SK^{313}	^{527}TG^{528}
	^{415}RK^{416}	^{634}SK^{635}	^{356}SK^{357}	^{90}TH^{91}
	^{578}RK^{579}	^{5}SV^{6}	^{418}SK^{419}	^{578}TK^{579}
	^{500}RL^{501}	^{33}SV^{34}	^{519}SK^{520}	^{636}TK^{637}
	^{570}RL^{571}	^{259}SV^{260}	^{259}SK^{260}	^{58}TL^{59}
	^{25}RM^{26}	^{368}SV^{369}	^{137}SW^{138}	^{552}TN^{553}
	^{20}RR^{21}	^{478}TD^{479}	^{377}TD^{378}	^{235}TR^{236}
	^{38}RR^{39}	^{552}TD^{553}	^{84}TE^{85}	^{343}TR^{344}
	^{7}RW^{8}	^{51}TE^{52}	^{139}TE^{140}	^{688}TR^{689}
	^{21}RW^{22}	^{84}TE^{85}	^{430}TE^{431}	^{40}TS^{41}
	^{285}SF^{286}	^{377}TE^{378}	^{582}TE^{583}	^{677}TS^{678}
	^{252}SH^{253}	^{557}TE^{558}	^{645}TE^{646}	^{212}TT^{213}
	^{33}SI^{34}	^{582}TE^{583}	^{663}TE^{664}	^{376}TT^{377}
	^{272}SK^{273}	^{645}TE^{646}	^{478}TG^{479}	^{577}TT^{578}
	^{312}SK^{313}	^{117}TG^{118}	^{527}TG^{528}	^{213}TV^{214}
	^{418}SK^{419}	^{139}TG^{140}	^{90}TH^{91}	^{547}TV^{548}
	^{519}SK^{520}	^{176}TG^{177}	^{578}TK^{579}	^{447}TW^{448}
	^{259}SV^{260}	^{527}TG^{528}	^{636}TK^{637}	^{656}TY^{657}
	^{137}SW^{138}	^{90}TH^{91}	^{58}TL^{59}	^{161}VD^{162}
	^{377}TD^{378}	^{636}TK^{637}	^{552}TN^{553}	^{239}VD^{240}
	^{139}TE^{140}	^{58}TL^{59}	^{235}TR^{236}	^{260}VD^{261}
	^{430}TE^{431}	^{102}TN^{103}	^{343}TR^{344}	^{314}VD^{315}
	^{582}TE^{583}	^{235}TR^{236}	^{688}TR^{689}	^{461}VD^{462}
	^{645}TE^{646}	^{577}TR^{578}	^{40}TS^{41}	^{607}VE^{608}
	^{663}TE^{664}	^{11}TS^{12}	^{677}TS^{678}	^{64}VF^{65}
	^{117}TG^{118}	^{40}TS^{41}	^{212}TT^{213}	^{214}VF^{215}

	^{478}TG^{479}	^{677}TS^{678}	^{376}TT^{377}	^{176}VG1^{77}
	^{527}TG^{528}	^{10}TT^{11}	^{577}TT^{578}	^{350}VG^{351}
	^{90}TH^{91}	^{376}TT^{377}	^{213}TV^{214}	^{537}VG^{538}
	^{10}TI^{11}	^{655}TT^{656}	^{547}TV^{548}	^{98}VK^{99}
	^{84}TK^{85}	^{213}TV^{214}	^{447}TW^{448}	^{209}VK^{210}
	^{636}TK^{637}	^{547}TV^{548}	^{656}TY^{657}	^{439}VK^{440}
	^{58}TL^{59}	^{447}TW^{448}	^{161}VD^{162}	^{543}VK^{544}
	^{327}TL^{328}	^{656}TY^{657}	^{239}VD^{240}	^{384}VL^{385}
	^{552}TN^{553}	^{161}VD^{162}	^{260}VD^{261}	^{426}VL^{427}
	^{343}TR^{344}	^{239}VD^{240}	^{314}VD^{315}	^{610}VL^{611}
	^{577}TR^{578}	^{461}VD^{462}	^{461}VD^{462}	^{410}VM^{411}
	^{688}TR^{689}	^{338}VE^{339}	^{607}VE^{608}	^{598}VS^{599}
	^{677}TS^{678}	^{214}VF^{215}	^{64}VF^{65}	^{57}VT^{58}
	^{212}TT^{213}	^{350}VG^{351}	^{214}VF^{215}	^{369}VT^{370}
	^{326}TT^{327}	^{537}VG^{538}	^{129}VG^{130}	^{581}VT^{582}
	^{376}TT^{377}	^{426}VH^{427}	^{176}VG1^{77}	^{666}VT^{667}
	^{213}TV^{214}	^{37}VK^{38}	^{350}VG^{351}	^{346}VW^{347}
	^{547}TV^{548}	^{209}VK^{210}	^{537}VG^{538}	^{548}VW^{549}
	^{447}TW^{448}	^{272}VK^{273}	^{98}VK^{99}	^{93}YA^{94}
	^{656}TY^{657}	^{430}VK^{431}	^{209}VK^{210}	^{227}YE^{228}
	^{239}VD^{240}	^{543}VK^{544}	^{439}VK^{440}	^{657}YE^{658}
	^{260}VD^{261}	^{384}VL^{385}	^{543}VK^{544}	^{189}YF^{190}
	^{314}VD^{315}	^{410}VL^{411}	^{384}VL^{385}	^{82}YG^{83}
	^{461}VD^{462}	^{548}VL^{549}	^{426}VL^{427}	^{524}YG^{525}
	^{64}VF^{65}	^{610}VL^{611}	^{610}VL^{611}	^{398}YI^{399}
	^{214}VF^{215}	^{260}VN^{261}	^{410}VM^{411}	^{72}YK^{73}
	^{350}VG^{351}	^{598}VS^{599}	^{598}VS^{599}	^{319}YL^{320}
	^{537}VG^{538}	^{34}VT^{35}	^{57}VT^{58}	^{324}YL^{325}
	^{98}VK^{99}	^{57}VT^{58}	^{369}VT^{370}	^{433}YL^{434}
	^{209}VK^{210}	^{546}VT^{547}	^{581}VT^{582}	^{588}YL^{589}
	^{338}VK^{339}	^{581}VT^{582}	^{666}VT^{667}	^{660}YL^{661}
	^{439}VK^{440}	^{666}VT^{667}	^{346}VW^{347}	^{20}YQ^{21}
	^{543}VK^{544}	^{346}VW^{347}	^{548}VW^{549}	^{192}YS^{193}

	^{607}VK^{608} ^{382}VL^{383} ^{384}VL^{385} ^{410}VL^{411} ^{426}VL^{427} ^{610}VL^{611} ^{475}VN^{476} ^{598}VS^{599} ^{57}VT^{58} ^{369}VT^{370} ^{581}VT^{582} ^{666}VT^{667} ^{346}VW^{347} ^{548}VW^{549} ^{93}YA^{94} ^{227}YE^{228} ^{657}YE^{658} ^{189}YF^{190} ^{82}YG^{83} ^{524}YG^{525} ^{398}YI^{399} ^{72}YK^{73} ^{135}YL^{136} ^{319}YL^{320} ^{324}YL^{325} ^{433}YL^{434} ^{660}YL^{661} ^{192}YS^{193} ^{665}YV^{666} ^{92}YY^{93} ^{523}YY^{524} ^{127}IPM^{129}	^{64}VY^{65} ^{81}VY^{82} ^{93}YA^{94} ^{65}YD^{66} ^{227}YE^{228} ^{657}YE^{658} ^{189}YF^{190} ^{82}YG^{83} ^{524}YG^{525} ^{324}YI^{325} ^{398}YI^{399} ^{400}YI^{401} ^{72}YK^{73} ^{319}YL^{320} ^{433}YL^{434} ^{660}YL^{661} ^{192}YS^{193} ^{665}YV^{666} ^{92}YY^{93} ^{523}YY^{524} ^{127}IPM^{129} ^{469}IPM^{471}	^{93}YA^{94} ^{227}YE^{228} ^{657}YE^{658} ^{189}YF^{190} ^{82}YG^{83} ^{524}YG^{525} ^{398}YI^{399} ^{72}YK^{73} ^{319}YL^{320} ^{324}YL^{325} ^{433}YL^{434} ^{588}YL^{589} ^{660}YL^{661} ^{20}YQ^{21} ^{192}YS^{193} ^{420}YS^{421} ^{665}YV^{666} ^{92}YY^{93} ^{523}YY^{524} ^{469}IPM^{471}	^{420}YS^{421} ^{665}YV^{666} ^{92}YY^{93} ^{523}YY^{524} ^{469}IPM^{471} ^{13}PP^{14} ^{373}MA^{374} ^{53}KA^{54} ^{12}SP^{13} ^{37}VR^{38} ^{15}EG^{16} ^{354}EH^{355} ^{416}ES^{417} ^{337}EV^{338} ^{355}HS^{356} ^{52}KK^{53} ^{512}LN^{513} ^{129}MG^{130} ^{128}PM^{129} ^{292}PQ^{293} ^{293}QG^{294} ^{259}SV^{260} ^{338}VK^{339} ^{127}IPM^{129}

	[469]IPM[471]			

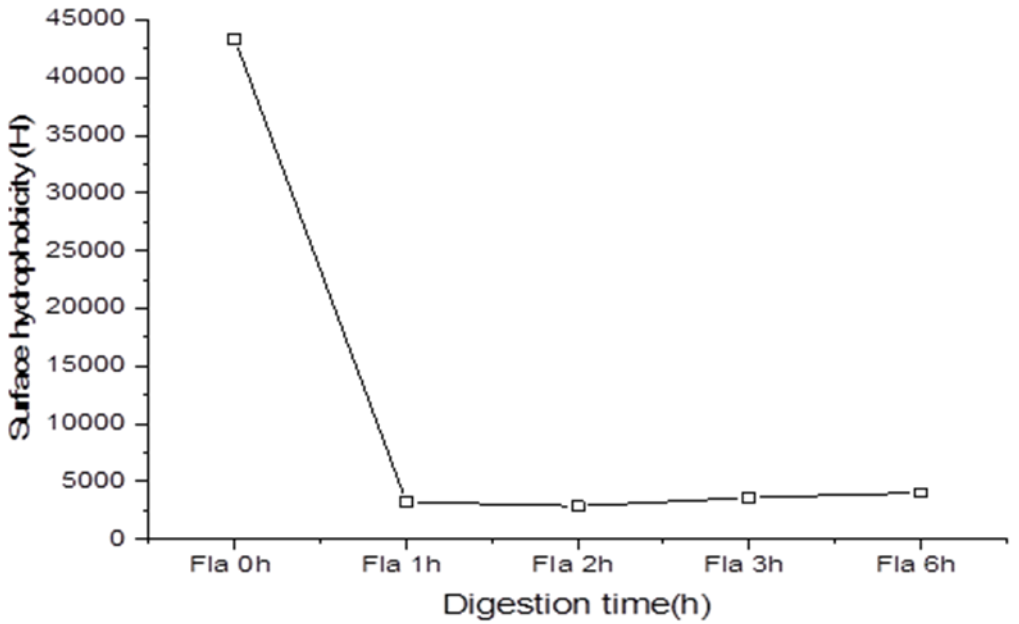


Figure S1. Protein surface hydrophobicity of the flavourzyme hydrolysate before, during, and after digestion by pepsin and trypsin.

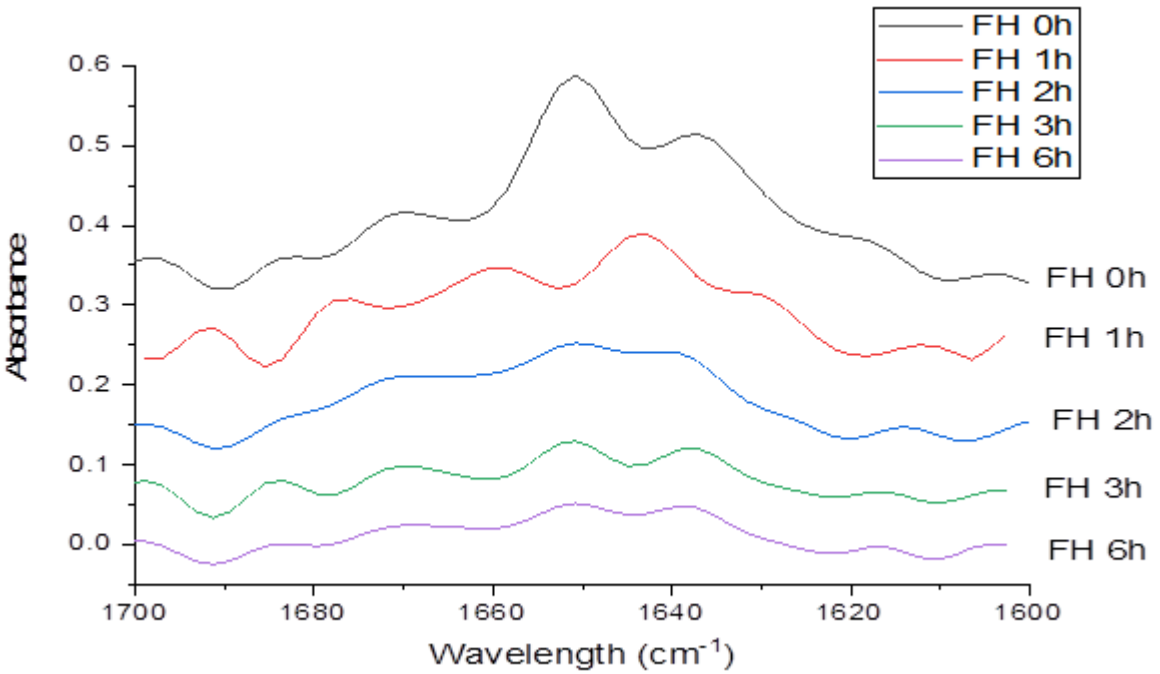
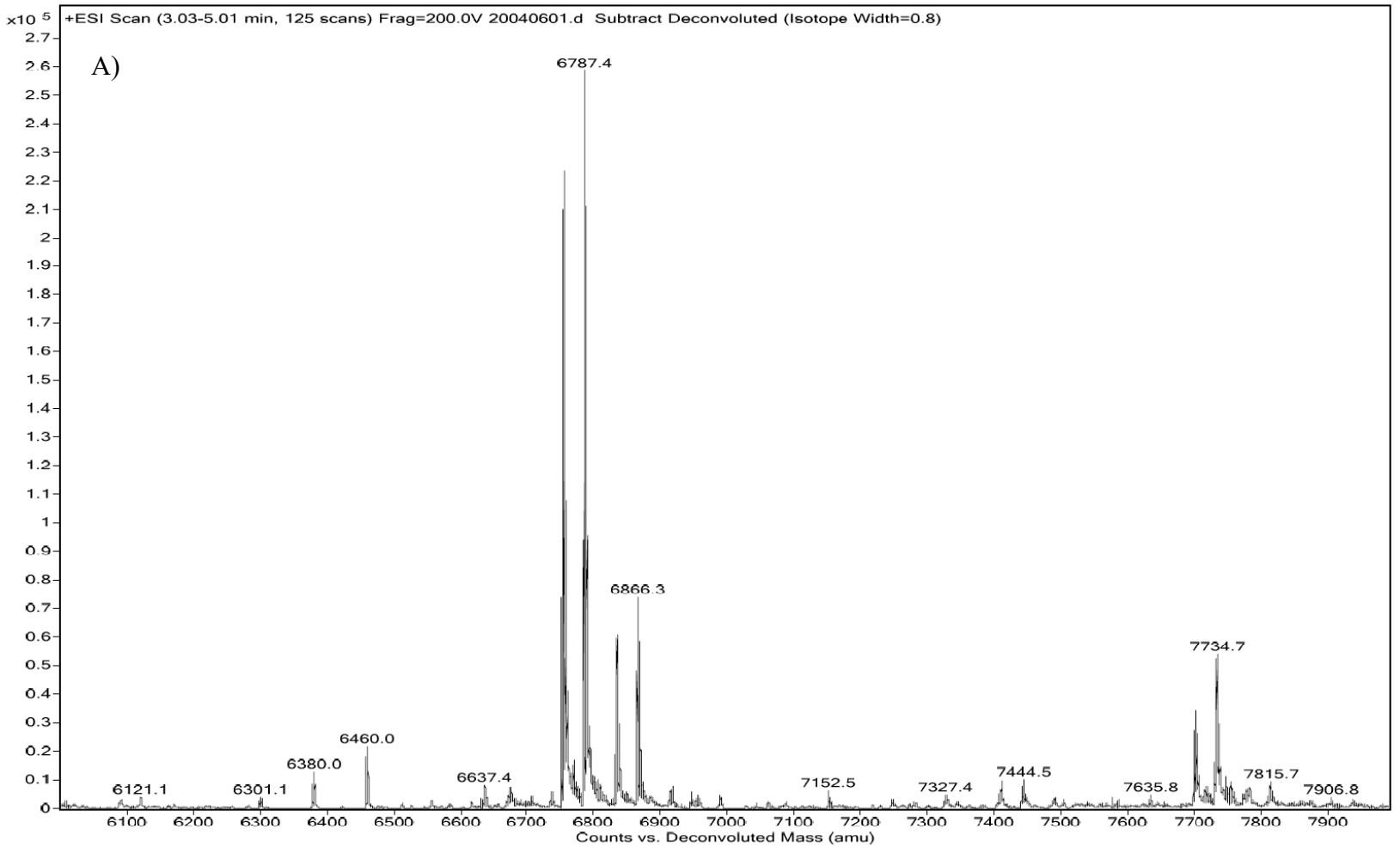


Figure S2. Conformation of peptide of the flavourzyme hydrolysate before, during, and after digestion by pepsin and trypsin.


+ESI Scan (3.03-5.01 min, 125 scans) Frag=200.0V 20040601.d Subtract Deconvoluted (Isotope Width=0.8)
x10 5
A)
6787.4
6866.3
7734.7
6460.0
6380.0
6637.4
6121.1
6301.1
7152.5
7327.4
7444.5
7635.8
7815.7
7906.8
Counts vs. Deconvoluted Mass (amu)

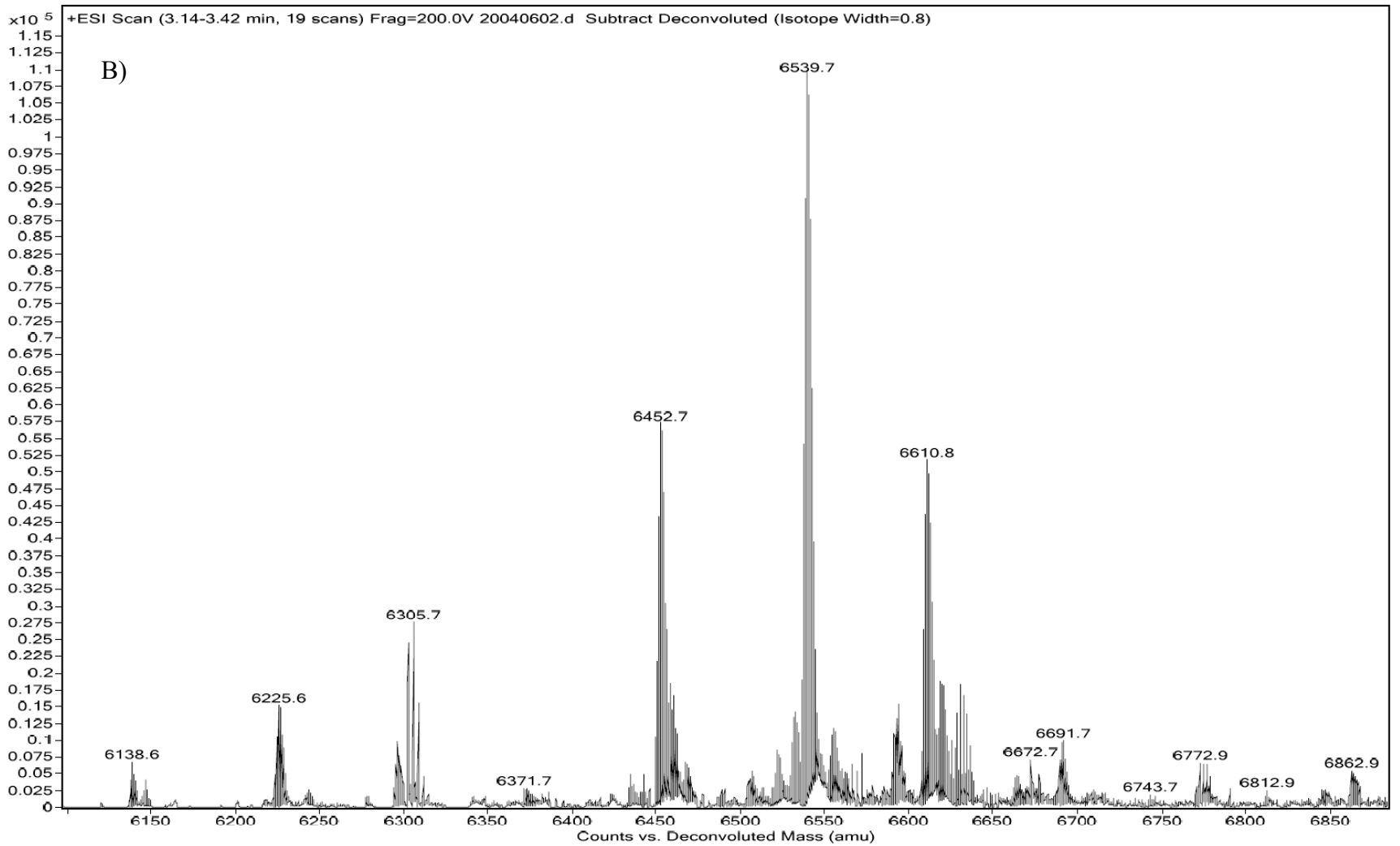


Figure S3: LC-MS analyses of bovine GMP (**panel A**) and GMP from Bactrian camels (**panel B)**