# Exploration of bioactive milk components from wild and domestic animals

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# 野生動物と家畜の乳に由来する生理活性物質の探索

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# Abbreviations

| | |
|---|---|
| $^{1}$H-NMR, | proton nuclear magnetic resonance spectroscopy; |
| ACE, | angiotensin converting enzyme; |
| ACN, | acetonitrile; |
| ANOVA, | analysis of variance; |
| ASD, | autism spectrum disorder; |
| CFM, | camel fermented milk; |
| CHCA , | α-cyano-4-hydroxycinnamic acid; |
| CMP, | cytidine monophosphate; |
| CN, | casein; |
| GDP, | guanindine diphosphate; |
| GLN, | glutamine; |
| GS/MS, | gas chromotography/mass spectrometry; |
| HFM, | horse fermented milk; |
| HPLC, | high performance liquid chromatography; |
| LAB, | lactic acid bacteria; |
| MALDI-TOF, | matrix-assisted laser desorption ionization time of flight; |
| MFGM, | milk fat globule membrane; |
| MRSA, | methicillin-resistant Staphylacoccus aureus; |
| MS, | mass spectrometry; |
| MS/MS, | tandem mass spectrometry; |
| MSSA, | methicillin-sensitive Staphylacoccus aureus; |
| NMWL, | nominal molecular weight limit; |
| PIC, | protease inhibitor cocktail; |
| PP3, | Lactophorin; |
| PTM, | post translational modification; |
| RP, | reversed phase; |
| SD, | standard deviation; |

| | |
|---|---|
| SDHB, | succinyl dehydrogenase B; |
| SER, | serene; |
| TFA, | trifluoroacetic acid; |
| UDP, | uridine diphosphate; |
| UV, | ultraviolet; |

# Chapter I

## General introduction

Despite potential bioactive components have been clarified from various proteins originated from animals and plants, mammalian milks are still major source of bioactive compounds (Hartmann & Meisel, 2007). The occurrence of bioactive components has already been reported in various species of milk and types of milk products, especially in the fermented milk products such as yogurt, sour milk and kefir (Hafeez *et al*, 2014). Moreover, traditional fermented milks that are produced by local people in the different part of the world have been considered to contain unknown bioactive compounds (Bayarsaikhan *et al*, 2011). In Mongolia, nomadic animal husbandry based on pasture production is the major industry which has been sustained for thousands of years in the continent. In order to satisfy the nutritional requirement of the animal, the nomads move with their livestock from place to place to cope up natural disaster during the drought and winter seasons. In general, seasonal movement of nomads is a strategy to escape sever feed shortage condition (Bat-Oyun *et al*, 2018). Nomadic people in Mongolia kept all of their animals including sheep, goats, cattle, horses and camels that are existed in nomadic regions all over the world and produce various products using their milks (Kurmann *et al*, 1992).

Horse (*Equus caballus*) and donkey (*Equus asinus*) are one of dairy species and belong to Equidae family. The domestication of these species about 5000 years ago was mostly for draught and transport purposes and had a big contribution on the development of rural societies (Beja-Pereira *et al*, 2004). Milks from these equids were believed to have functional and therapeutic properties, and donkey's milk is mainly used in several African countries (Pearson *et al*, 2005) while fermented mare's milks are traditionally produced and consumed in Eurasian steppe areas. In this regard, Mongolia is a top producer of horse milk and it is mainly used for *airag* production, reported with 100 thousand ton in 2011 (Tsetsgee & Damdinsuren, 2012). It has been roughly estimated that Mongolian nomads consume ab1o1ut 60 kinds of traditional livestock dairy products

and essential foods (Bayarsaikhan *et al*, 2011). Generally, several type of fermented milk products are available in local areas of Mongolia. The most famous fermented products are *"khoormog"* and *"airag"* made by camel's and mare's milks, respectively. There have been several reports on traditional methods owned by Mongolian nomads for manufacturing dairy products including camel's and mare's milks (Konagaya 1996; Ishige 1997; Takahashi, 2000a; 2000b; Ishii & Samejima, 1999; Ishii, 2000; Adachi, 2002). In a restricted desert area in Mongolia, where camels are fed, fermented camel's milk production and consumption are popular (Ishii & Samejima, 2006), whereas fermented mare's milk intensively produced and consumed in central, western and southern Mongolia (Bat-Oyun *et al*, 2015). Furthermore, Mongolian nomads believe that especially fermented camel's and mare's milks have pharmaceutical activities. For example, the fermented camel's milk has been used to treat edema in pregnant women and as anti-scorbutic agent for the elderlies during the seasonal changes in Mongolia (Dubach *et al*, 2007), while fermented mare's milk have positive effect to improve the immune system and for treatment of lung and heart disease (Wang *et al*, 2008; Yuan *et al*, 2006).

There are two domesticated species of camel: one-humped, dromedary camel (*Camelus dromedaries*) and two-humped, Bactrian camel (*Camelus bactrianus*). Total population of dromedary and Bactrian camels was about 28 million head in 2015 according to FAO statistic (FAO, 2015). An estimated global camel milk output is about 5.3 million tons (FAO/CIRAD/KARKARA), however the available worldwide camel milk production has been assessed at 1.3 million tones (FAO, 2008), the values estimated under pastoral conditions suggesting that more than 75% of camel's milk is consumed by nomads as raw milk and milk products. Somalia was highest producer of camel's milk (0.85 million tons per year), accounting for more than 50% of the available camel's milk production in the world by 2005. A variety of traditional dromedary camel's milk products exist despite difficulty in developing milk preservation techniques under the hot and dry desert environments. These include dried curd from fermented milk (*oggt*) in Saudi Arabia, fermented milk (*susa*) in East Africa, clarified fat (*ghee*) in Somalia, and fermented camel's milk (*shubat)* in Kazakhstan (Konuspayeva *et al*, 2004), *suucas*

in Kenya (Lore *et al*, 2005), *gariss* in Sudan (Sulieman *et al*, 2006), and butter (*shmen)* in Algeria (Kacem & Karam, 2006).

Although dromedary camel's milk has been reported as being suitable for drinking (Yagil *et al*, 1984), several attempts on milk processing under laboratory conditions have been made for the production of butter (Farah *et al*, 1989; Berhe *et al*, 2013), ice cream (Abu-Lehia *et al*, 1989; Soni & Goyal, 2013), soft cheese (Mehaia, 1993; Konuspayeva *et al*, 2014), and yogurt (Hashim *et al*, 2009; Varga *et al*, 2014; Al-Zoreky & Al-Otaibi, 2015). Recently, "Al Nassma Chocolate" company in the United Arab Emirates has started commercial production of chocolates containing camel's milk.

**I-1. Camel's and mare's milks compositions and their roles in nutrition**

Milk is a complex biological fluid secreted by mammalian udders, as a first food for newborns, and provides the excellent source of nutrients needed for their proper growth and development (Park, 2009). Milk composition varies among animal species and depending on the lactation period. For example, Bactrian camel's milk contains approximately 3.5% proteins, 4.4% fat, 4.9% lactose, 13.6% dry matter and 0.7% ash (Indra, 2003). On the other hand, Mongolian horse milk contains 2.2% proteins, 2.4% fat, 7.2% lactose and 10,6% dry matter , as reported by Naidankhuu & Dag Austbo (2010).

**I-1-1. Carbohydrates**

Lactose, Gal ($\beta$1- 4) Glc, is a major carbohydrate in milk, accounting for 2.4-5.8% of total solids in Bactrian and dromedary camel's milks (Konuspayeva *et al*, 2009). Zhang *et al*, (2005) reported that the average concentration of lactose in Alxa Bactrian camel's milk to be 4.44%. The lactose concentration in camel's mature milk was almost constant during the different stages of lactation (Hassan *et al*, 1987; Zhang *et al*, 2005), but dehydration occurred by lack of water intake leads to decreases in lactose content to 2.9% (Yagil & Etzion, 1980). Mare's milk contains high amount of lactose (6.4%) compare to cow's and camel's milks (Pieszka *et al*, 2016).

In addition to lactose, small amounts of variety of oligosaccharides, of which the reducing end is usually composed of a lactose unit, are contained in mammalian milk (Urashima *et al*, 2011). Milk oligosaccharides are believed to play significant roles in protection against pathogens, promotion of bifidus flora formation, and development of the nervous system, whereas lactose is an important energy source for neonates (Urashima *et al*, 2009). Fukuda *et al*. (2010) identified the presence of lactose, lacto-*N*-neotetraose, lacto-*N*-novopentaose I, and lacto-*N*-neohexaose, known as type II structure, found in colostrum were core structures of Bactrian camel's milk oligosaccharides. Alhaj *et al* (2013) found type II oligosaccharides in commercially pasteurized dromedary camel's milk, as same as Bactrian camel's milk, indicating a high similarity in milk oligosaccharide profiles between dromedary and Bactrian camels. These characteristics were more similar to bovine milk than human milk (Marino *et al*, 2011). Recently, comparative analysis has been conducted by Albrecht *et al*, (2014) on milk oligosaccharides of several domestic animals. The fact that higher content of sialyl oligosaccharides in camel's colostrum than in cow's colostrum may give an opportunity to camel's colostrum as a better commercial source of sialyl oligosaccharides (Fukuda *et al*, 2010).

**I-1-2. Proteins**

In Bactrian and dromedary camel's milks, total protein concentrations are 2.6-4.8 and 2.2-4.9 g/L, respectively (Konuspayeva *et al*, 2009). Mare's milk protein concentration is about 2.1 g/L (Pieszka *et al*, 2016). Milk proteins are classified into caseins and whey proteins. Characteristics of major milk proteins are described in detail as below.

**I-1-2-1. Caseins**

Caseins ($\alpha_{s1}$, $\alpha_{s2}$, $\beta$ and $\kappa$) are the major proteins in mammalian milk, but their total quantity and portion varies depend on animal species. Caseins in mature milk of humans, cows, and dromedary camels account for about 45, 80, and 70% of total proteins, respectively. Casein fraction in non-fat milk of Alxa Bactrian camels was 30.9$\pm$2.9% of total protein at 2 hours after parturition and increased gradually during lactation, reaching 52.2$\pm$0.2% at 90 days after birth (Zhang *et al*,

2005), which is similar to that observed in human milk. Bonnizi *et al*, (2009) reported that the ratio of $\alpha_{s1}$ : $\alpha_{s2}$ : β : κ-casein in cow milk is 37 : 6.1 : 44.2 : 12.7, whereas the ratio in dromedary camel's milk is 22 : 9.5 : 65 : 3.5 (El-Agamy, 2009) and in mare's 17.9 : 1.4 : 78.5 : 1.8 (Salimei & Fantuz, 2012). The β-casein is predominant in cow and dromedary camel's milks, however, its ratio in the total proteins in dromedary camel's milk is markedly higher than that in cow milk and this trend is similar to that in human milk. The content of κ-casein is significantly lower in milks of dromedary camel and mare than that of cow (Farah-Riesen, 1985; Salimei *et al*, 2004).

**I-1-2-2. Whey proteins**

El-Hatmi *et al*, (2007) has been reported that immunoglobulins are the most abundant proteins in dromedary colostral whey (101.8 g/L), but this amount rapidly decreased to 19.6 g/L at 48 hours after parturition and reaches 7.9 g/L at 192 hours after birth, which is similar to that in cow milk. In mature milks of Bactrian (Zhang *et al*, 2005) and dromedary camels (El-Hatmi *et al*, 2006; El-Agamy *et al*, 2009), α-lactalbumin and blood serum albumin are the dominant whey proteins. In Bactrian and dromedary camels' milks, whey contain three and two α-lactalbumin variants, respectively (Ochirkhuyag *et al*, 1998). Compared with cow milk, a prominent characteristic of Bactrian and dromedary camels' milks is an absence of β-lactoglobulin, which is the main component (50%) of cow whey proteins (Ochirkhuyag *et al*, 1997; Merin *et al*, 2001; Kappeler *et al*, 2003; Zhang *et al*, 2005; El-Hatmi *et al*, 2007; Hinz *et al*, 2012; Zhao *et al*, 2015). Lactoferrin is an 80-kDa glycoprotein, a member of the transferrin family. It displays a wide array of protective effects, such as anti-inflammatory, antimicrobial, antitumor, and immunomodulatory activities. The lactoferrin concentration in mature milk of dromedary camel ranges from 0.02 to 2.1 g/L (Al-Majali *et al*, 2007). Similar trend of lactoferrin concentration in Bactrian camel (0.22 ± 0.132 g/L) and in dromedary milk (0.209 ± 0.131. g/L) was reported by Konuspayeva *et al*, (2007).

**I-1-3. Lipids**

The lipid profile of camel's milk is known to be different from those of various mammalian species. The fat content of camel's milk is comparable to that of cow's milk and usually ranges from 1.2 to 6.4% (Konuspayeva *et al*, 2009). However, as compared to cow milk, short-chain fatty acids and carotene contents are lower in camel's milk (Abu-Lehia, 1989; Stahl *et al*, 2005). Similarly, a concentration of cholesteryl ester fatty acids was slightly lower in camel's milk (52%) than in cow's milk (58%), but in both cases, palmitic acid was identified as the major fatty acid (Gorban & Izzeldin, 1999). Electrospray ionization tandem mass spectrometry analysis demonstrated that 74% of the total fatty acid among the triacylglycerols in dromedary camel's milk was composed of one of the four major fatty acids, C14:0, C16:0, C18:0, or C18:1 (Haddad *et al*, 2011). The above study also found that 75% of such fatty acids contain at least an unsaturated fatty acid. In a comparative analysis of fatty acids using silver ion solid phase extraction and GC/MS methods, Dreiucker & Vetter (2011) found that *iso*- and *anteiso*-fatty acid contents were higher in camel's milk as compared to those in milks from cow, moose, and human. Long-chain fatty acids, which may reduce the lipids in human blood, are abundantly found in camel's milk as well as unsaturated fatty acids. However, the content of such fatty acids may vary among milk from camels in different geographical regions (Ereifej *et al*, 2011; Konuspayeva *et al*, 2008). Furthermore, fatty acid contents are also different between dromedary and Bactrian camel's milk. For instance, Konuspayeva *et al*. (2008) found that the proportion of C17:0 *iso* and C18:1 fatty acids was higher in dromedary camel's milk than that in milk from Bactrian camel. Additionally, phospholipid content in camel's milk (0.503 mM) as determined by $^{31}P$ nuclear magnetic resonance was higher than that in milks from mare, human, and cow (0.101, 0.324 , 0.265 mM, respectively) (Garcia *et al*, 2012). Because of the variations in lipid profiles and other properties between camel's milk and human's milk, the former cannot be a direct substitute for the later. Therefore, supplementation of polyunsaturated fatty acids and fats and minor lipids containing 1,3-dioleoyl-2-palmitoylglycerol is very important when camel's milk is used for preparation of infant formula (Zou *et al*, 2013).

### I-1-4. Mineral salts and vitamins

In general, the major mineral concentrations in camel's and mare's milks are rather similar to those in cow's milk. Bactrian camel's milk contains higher concentrations of phosphorus than in dromedary milk (Faye *et al*, 2008). It is important to know that the calcium to phosphate ratio in milk given to human newborn infants is appropriate to prevent the hyperphosphatemia and hypocalcemia (Gittleman & Pincus, 1951; El-Agamy, 2006). Camel's milk is an excellent source of iron and can support quick growth in infants and protect them from iron-deficiency anemia. Chloride is also rich in dromedary camel's milk as a result of forage diet intake, e.g, *Atriplex* and *Acacia* (Khaskheli *et al*, 2005). The advantageous lower level of citrate (128 mg/dL) in camel's milk enhance the antimicrobial activity of lactoferrin (El-Agamy, 2006).

Camel's milk is obviously a good source of vitamin B complex and C. In particular, one of benefitial aspects of camel's and mare's milks is that contains higher concentrations of vitamin C than in cow and human milks (Sheng & Fang, 2009). Although a wide variations in vitamin concentrations of camel's milk owing to bred, feed, stage of lactation, and so on, it is likely that concentrations of vitamins A, D, and E in milk are higher in Bactrian that in dromedary camel (Zhang *et al*, 2005).

## I-2. Health-beneficial aspects of camel's and mare's milks and their products

In the milk, certain amount of peptides exist, most of bioactive peptides are encrypted in milk proteins in inactive form, and those peptides are released by the action of proteolytic enzymes during either fermentation of dairy foods or digestion in the gastrointestinal tract of consumers (Meisel & Bockelmann, 1999; Korhonen & Pihlanto, 2006). Milk derived bioactive peptides are particular interest in food science and nutrition because they show physiological and biological function such as opioid-like (Chang *et al*, 1981), immunomodulating (Fiat *et al*, 1993), antitumor (Korashy *et al*, 2012), antibacterial (Bellamy *et al*, 1992), antidiabetic (Sboui *et al*, 2012), anti-oxidant (Halima *et al*, 2014), and antihypertensive activities (Hata *et al*, 1996) as explained below.

### I-2-1. Antimicrobial activity

Camel's lactoferrin showed highest bactericidal activity against enterohemorrhagic *Escherichia coli* O157, H7 when compared with sheep, goat, alpaca, elephant and a human lactoferrin (Conesa *et al*, 2008). Redwan *et al*, (2014) reported camel's lactoferrin and its digested N- and C-lobes to possess potential anti-infectious activities against hepatitis C virus. Concentration of another antimicrobial agent, lysozyme, has been reported to be higher in dromedary camel's milk (150 µg/L) compared to cow milk (70 µg/L) (El-Agamy *et al*, 1998). Immunoglobulin exhibits low antibacterial activity but inhibits the proliferation of rotavirus effectively (El-Agamy *et al*, 1992). Fermented camel's milk was reported to show antibacterial activity mainly due to the production of bactericidal peptides due to action of exogenous proteinases (Jrad *et al*, 2014; Zeineb *et al*, 2015). *Lactobacillus acidophilus* AA105 isolated from camel's milk produces a bacteriocin-like substance, which induces *Listeria monocytogenes* cell lysis (Abo-Amer, 2013). In 2011, Tidona *et al* (2009) reported that anitimicrobial activity of donkey's milk was also a result of peptides generated during the gastrointestinal digestion of milk protein. Antibacterial effect of fermented mare's milk (*kumis*) was enhanced by the presence of probiotics, such as *L. acidophilus* and *B. bifidum* as well as yeasts (Pieszka *et al*, 2016).

### I-2-2. Antitumor activity

A Middle East legend stating camel's milk to be beneficial for the prevention and treatment of cancer was recently supported by the results of study conducted by Korashy *et al*, (2012). They reported that inhibition in proliferation of human hepatoma (HepG2) and human breast cancer cell line (MCF7) by dromedary camel's milk through the activation of pro-apoptotic caspase-3 expression and the induction of death receptors, probably resulting in antitumor effects. *In vitro* experiments also suggest camel's milk lactoferrin being capable of inhibiting proliferation of a colon cancer cell line, HCT-116 (Habib *et al*, 2013). Further, liposomes prepared from camel's milk phospholipids mixture were documented as potential carriers of anticancer drugs (Maswadeh *et al*, 2015). The *in vitro* effect of antitumor compounds have been recently observed in donkey milk (Mao *et al*, 2009).

### I-2-3. Antidiabetic activity

A hypoglycemic effect of dromedary camel's milk or its fermented products, *shubat*, has been demonstrated in animal (Sboui *et al*, 2012, Manaer *et al*, 2015) and human patients (Agrawal *et al*, 2013; Ejtahed *et al*, 2015). Korish (2014) reported that molecular mechanism of hypoglycemic activity of camel's milk included changes in glucagon like peptide-1, glucose dependent insulin-tropic peptide, glucose tolerance, fasting and glucose-stimulated insulin secretion, insulin, resistance, tumor necrosis factor-α (TN-α), transforming growth factor-β1 (TGF-β1), lipid profile, atherogenic index, and body weight. Oral administration of camel's milk ameliorated diabetic symptoms, including reduction of homeostasis model assessment-insulin resistance and the levels of incretin hormones, TNF-α and TGF-β1 elevated in the type 2 diabetes model rat (Korish, 2014).

### I-2-4. Antihypertensive activity

Camel's milk proteome is supposed to be a rich source of novel bioactive peptides along with the known ones. Alhaider *et al*. (2013) reported presence of blood pressure-lowering peptides using *de novo* sequencing of peptides by mass spectrometry. In fact, several angiotensin converting enzyme (ACE) inhibitory peptides capable of lowering blood pressure were found in a *Lactobacillus rhamnosus* PTCC 1637 fermented product of dromedary camel's milk by Moslehishad *et al*, 2013. Further, the peptide fraction of fermented camel's milk showed higher ACE inhibitory activity compared to fermented bovine milk, suggesting the fermented camel's milk as a highly potential functional food with antihypertensive effects (Moslehishad *et al*, 2013). ACE inhibitory activity has also been reported by Jrad *et al* (2014) in enzymatic digestives of camel's colostrum, colostral whey, and milk proteins. By the simulated gastrointestinal digestion of donkey's milk, an ACE inhibitory peptide from β-casein has been identified recently (Bidasolo *et al*, 2012).

### I-2-5. Antioxidant activity

It is well documented in literature that antioxidant activity of camel's milk is due to α-lactabumin (Halima *et al*, 2014), lactoferrin (Habib *et al*, 2013) and casein hydrolysates (Al-Saleh *et al*, 2014; Jrad *et al*, 2014). It provides corroborative evidences for the potential use of camel's milk in colon cancer therapeutics (Habib *et al*, 2013), diabetes (Ebaid *et al*, 2013; Korish *et al*, 2015), alcohol-induced liver injury (Darwish *et al*, 2012), non-alcoholic fatty liver disease (Korish & Arafah, 2013), and autism spectrum disorder (ASD) (Al-Ayadhi & Elamin, 2013). Ghamari *et al*. (2013) reported that camel β-casein and its peptides synergistically enhanced the antioxidant activity of aloin, a major active phenolic components of *Aloe vera.*

**I-2-6. Immunomodulating avtivity**

Oral administration of dromedary camel's milk significantly ameliorated 2,4,6-trinitrobenzene sulfonic acid-induced colitis in rats, through reduced myeloperoxidase activity and colonic levels of tumor necrosis factor (TNF)-α and interleukin (IL)-10, suppression of oxidative stress, stimulation of antioxidant activity, and inhibition of caspase-3 activity (Arab *et al*. 2014). Alhaider *et al*. (2014) reported that oral gavage of camel's milk downregulated pro-angiogenic and pro-inflammatory cytokines production, such as vascular endothelial growth factor, IL-1β, IL-6, IL-17, TNF-α, and transforming growth factor-β, in mice, resulting in inhibition of inflammatory angiogenesis, an important phase in the tumor development and metastasis.

Un-denatured whey proteins in dromedary camel's milk have been shown to have an effect on host immune system. Oral administration of the whey protein fraction to mice reportedly increased the serum levels of IL-2 and IL-8 and decreased the levels of pro-inflammatory cytokines, IL-1α, IL-1β, IL-6, and IL-10 (Ebaid *et al*, 2012). This immunomodulating activity of whey proteins was further elucidated for cellular and humoral immunities using streptozotocin-induced diabetic rodents, especially in relation to alleviation of wound healing process (Badr, 2012; Badr *et al*, 2012; Ebaid *et al*, 2013; Ebaid, 2014). It was also reported that the induction of β-defensins due to stimulation of host immune system underlines the promotion of diabetic wound healing in the streptozotocin-induced diabetic mouse model (Badr, 2013).

A double-blind clinical trial performed by Bashir & Al-Ayadhi (2014) to assess immunomodulating activity of camel's milk in autistic children suggested that after two weeks consumption, the serum levels of thymus and activation-regulated chemokine, which is a specific chemoattractant of T lymphocyte, were significantly reduced in boiled (P=0.004) and raw (P=0.01) camel's milk treated groups compared to placebo group (P=0.68). Significant improvement in the score of childhood autism rating scale was reported only in the raw camel's milk treated group (Bashir & Al-Ayadhi, 2014). This data suggests the potential of camel's milk as a promising therapeutic agent in alimentary treatment of Autism spectrum disorder.

**I-3. Significance of the study**

In general, mammalian colostrum and mature milk contain lactose as the predominant carbohydrate together with minor amount of a variety of oligosaccharides, most of which contain a lactose unit at their reducing ends. The milk oligosaccharides have been studied in colostrum and milk of Bovidae species such as cows, sheep and goats. In these species, many different chemical structures of neutral and acidic oligosaccharides have been identified and characterized up to date. That is to say, a heterogeneity of milk oligosaccharides have been observed among Bovidae species. Milk oligosaccharide structures of domesticated Bovidae species have been well characterized, whereas those in wild Bovidae species are still unclarified (Sasaki *et al*, 2016). To explore novel biofunctional compounds, especially milk oligosaccharides not only from livestock animals but also from wildlife is significant sources. Once we identified and characterized the novel oligosaccharides from colostrum and mature milk of wild animals and it will be useful for biofunctional ingredients in food and medical factory. Oligosaccharides in the milk or colostrum of the addax (*Addax nasomaculatus*), an Antelope species of Bovidae, have not so far been clarified. Therefore, present study will focus on characterization of sialyl oligosaccharides, a highly potential candidates for bioactive components, in colostrum of addax in comparison to those found in cows, sheep and goats.

Camel's and mare's milks are one of valuable sources of essential nutrients. Besides, they contain bioactive peptides that have functionality and medicinal properties to human health

(Kaskous, 2016 & Pieszka *et al*, 2016). Regarding to bioactive peptides originated from camel's and mare's milks proteins, most of them are still unknown. In addition, many studies have been revealed that the fermentation process have improved the biological activities of camel's and mare's milks. From this point of view, a couple of comprehensive studies on dromedary camel's milk and its fermented product have been performed. Up to date, there is lack of information on peptides naturally occurred in fermented dairy products produced by traditional method. Therefore, peptides isolation from fermented Mongolian camel's and mare's milks and investigation of their antimicrobial activity will be concluded in the present study.

**I-4. Objectives of this study**

The objectives of this study are to explore bioactive compounds from wild and domestic animals: the first is characterization of sialyl oligosaccharides in colostrum of addax and compare with those of cow, sheep and goat, the second is isolation and identification of peptides in fermented camel's and mare's milks produced by traditional method in Mongolia, and the third is evaluation of antimicrobial activities of whey ultra-filtrates and peptide fractions obtained from fermented camel's and mare's milks.

# Chapter II

## Identification of sialyl oligosaccharides including an oligosaccharide nucleotide in colostrum of an addax (*Addax nasomaculatus*) (Subfamily Antelopinae)

### II-1. Introduction

Mature milk and colostrum from mammalian usually contain lactose (Gal(β1-4)Glc) as the major carbohydrate as well as minor quantity of an array of oligosaccharides, most of which contain a lactose unit at their reducing ends (Jenness *et al*, 1964; Urashima *et al*, 2007; 2011; 2014; 2016). It has been elucidated that mature milk or colostrum of a few species including sheep (Sasaki *et al*, 2016), reindeer (Taufik *et al*, 2014), pig (Kobata *et al*, 1965) and human (Kobata, 1963; 1966), in addition, contain oligosaccharide nucleotides. Gal(β1-4)GlcNAc-α1-uridine diphosphate *N*-acetyllactosamine has been found in milk of pig (Kobata *et al*, 1965), human (Kobata, 1963; 1966) and reindeer (Taufik *et al*, 2014), while Fuc(α1-2)Gal(β1-4)GlcNAc-α-1-UDP has been identified in human milk (Kobata, 1963; 1966). Ovine colostrum contains Neu5Gc(α2-6)Gal(β1-4)Glc-α1-UDP and Neu5Gc(α2-3)Gal(β1-4)Glc-α1-UDP (Sasaki *et al*, 2016). It has been published that caprine colostrum contains Sia(α2-3(6))Gal(β1-4)GlcNAc-α1-UDP and Sia(α2-3(6))Gal(β1-6)GlcNAc-α1-UDP (Jourdian *et al*, 1961). Usually, sugar nucleotides such as UDP-Gal, UDP-GlcNAc, cytidine monophosphate-Neu5Ac, guanidine diphosphate-Fuc etc., function as donors for glycolsyltransferases that transfer monosaccharides to acceptor glycolconjugates within the Golgi apparatus of cells. Although the those of UDP-oligosaccharides could theoretically be utilized as donors for enzymes that transfer LacNAc, fucosyl LacNAc (Fuc(α1-2)Gal(β1-4)GlcNAc) or sialyl LacNAc to suitable acceptors, such glycolsyltransferases have never been detected in any biological sources, especially in the mature milk and colostrum. In order to clarify the biological significance of oligosaccharides nucleotides in mature milk and colostrum, it needs to explorer which species' mature milk and colostrum contain such components. In this study, I focused on colostrum of Artiodactyla for this purpose, because reindeer and sheep, whose mature

milk and colostrum contain UDP-LacNAc or UDP-sialyl LacNAc (Sasaki *et al*, 2016; Taufik *et al*, 2014), are both Artiodactyla species. As a result, a UDP-sialyl LacNAc was characterized as well as sialyl oligosaccharides in acidic saccharide fraction, separated from the colostrum of addax (*Addax nasomaculatus*), an Antelopinae species among Artiodactyla (Fig. 1).

## II-2. Materials and methods

### II-2-1. Colostrum sample

Colostrum sample (16 mL) was collected from a lactating addax bred at Himeji Central Park, Japan. The sample was collected manually at 50 hours post partum on 3rd of April, 2016.

### II-2-2. Oligosaccharide standards

Uridine 5'-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc), uridine 5'-diphospho-*N*-acetyl-D-galactose (UDP-Gal), Neu5Ac(α2-3)Gal(β1-4)Glc (3'-SL), Neu5Ac(α2-6)Gal(β1-4)Glc (6'-SL) and Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc (di-*N*-acetylneuraminyl lactose) were purchased from Sigma Co. (St. Louis, MO, USA).

### II-2-3. Experimental methods

#### II-2-3-1. Isolation of acidic saccharides from addax colostrum

The milk sample was thawed and 15 mL were treated and mixed with four volumes of chloroform/methanol (2:1, v/v), after which the resulting emulsion was centrifuged at 4°C and 4,000 x *g* for 30 min. The lower layer containing chloroform and denatured protein were discarded. The upper layer was concentrated to 10 mL by rotary evaporation, which were then treated with three volumes of ethanol, and allowed to stand overnight at 4°C. The resulting precipitate was removed by centrifugation at 4°C and 4,000 x *g* for 30 min, the supernatant was concentrated to dryness by rotary evaporation, and the residue dissolved in 5 mL of water and lyophilized. The lyophilized material, designated the carbohydrate fraction, was dissolved in 4 mL of water and separated into seven fractions (AC-1 to AC-7, Fig. 1) by gel filtration with a BioGel P-2 column (45 μm, ϕ 2.5 × 100 cm). The gel had been throughly washed with 0.1 M HCl and 0.1 M NaOH

and equilibrated with water before use. Elution was done with distilled water at a flow rate of 15 mL/h, and fractions of 5 mL were collected. Each fraction was analyzed for hexose with phenol-$H_2SO_4$ method (Dubois *et al,* 1956) and for sialic acid with periodate-resorcinol method (Jourdian *et al,* 1971). Peak fractions were pooled and lyophilized.

The components in AC-1 to AC-4 were separated by high performance liquid chromatography (HPLC) on a TSK gel Amide-80 column (ϕ 4.6 × 250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan) using a LC-10 ATVP pump (Shimadzu, Tokyo, Japan) (Fig. 2). The mobile phase was 50% and 80% (vol/vol) acetonitrile ($CH_3CN$) in 15 mmol/L potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60 °C at a flow rate of 1 mL/min. The eluates were monitored by measuring the absorbance at 195 nm. The peak fractions of the saccharides were pooled, concentrated by rotary evaporation, and subjected to $^1$H-nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to characterize their structures.

**II-2-3-2. Proton nuclear magnetic resonance spectroscopy ($^1$H-NMR)**

NMR spectra were recorded in $D_2O$ (99.999 atom %D, Acros Organics, New Jersey, USA) at 500 or 600 MHz for $^1$H-NMR with a JEOL ECP-500 Fourier transform-NMR (Jeol, Tokyo, Japan) or a Varian INOVA 600 spectrometer (Varian Inc., Palo Alto, CA, USA) operated at 293.1 K. Chemical shifts were expressed as change relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid, sodium salt, but were actually measured by reference to internal acetone (δ = 2.225).

**II-2-3-3. Mass spectrometry**

MALDI-TOF MS was performed on the oligosaccharide fractions, using an Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Lyophilized oligosaccharide fractions were dissolved in 5 μL of milli-Q water. The oligosaccharide solution was mixed with an equal volume of 10 mg/mL SDHB (Bruker Daltonics), which is a mixture of 2,5-dihydrobenzoic acid and 2-hydroxy-5-methoxybenzoic acid, on a target plate (MTP 374 ground steel TF target

plate, Bruker Daltonics), and dried at ambient temperature. After the solvent dried, the target plate was loaded into the mass spectrometer. Mass spectra were obtained using a reflector positive ion mode optimized to mass range of 0-3 kDa. Peptide calibration standard II (Bruker Daltonics) was used for external calibration of the mass spectrometer.

**II-3. Results**

**II-3-1. Separation of oligosaccharides and oligosaccharide nucleiotides from addax colostrum**

As shown in Fig. 2, carbohydrate fraction isolated from the addax colostrum was separated by gel filtration on BioGel P-2 into at least seven peaks, designated as AC-1 ~ AC-7. As given a positive result with the periodate-resorcinol method, the components in AC-1, AC-2, AC-3 and AC-4 were assumed to be acidic saccharides. According to our focus in this study, to explore sugar nucleotides, acidic saccharides were further subjected to normal phase HPLC with a TSK Amide-80 column. Based on the eluted positions, it was thought that fraction AC-5, AC-6 and AC-7 contained neutral oligosaccharides, lactose and monosaccharides, respectively. Following size-exclusion chromatography on BioGel P-2, acidic fractions of AC1, AC2, AC3 and AC4 were separated by HPLC into several peaks designated as below and shown in Fig. 3: AC1 into AC1-1 ~AC1-10 (Fig. 3A), AC2 into AC2-1~AC2-6 (Fig. 3B), AC3 into AC3-1~AC3-4 (Fig. 3C) and AC4 into AC4-1~AC4-7 (Fig. 3D), respectively. Those fractions purified by HPLC were then characterized by $^{1}$H-NMR and MALDI-TOF MS.

**II-3-2. Structural determination of isolated oligosaccharides and oligosaccharide nucleotides**

**II-3-2-1. Assignment of $^{1}$H-NMR spectrum obtained from AC1-2.**

$^{1}$H-NMR spectrum (chemical shifts in Table 1) of the oligosaccharide in AC1-2 had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.223, 4.663 and 4.521, respectively. The $^{1}$H-NMR spectrum had the H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac at δ 1.751 and 2.778, respectively, and the H-3 axial and equatorial shifts of α(2-8) linked Neu5Ac at δ 1.731 and 2.714, respectively (Fig. 4). The spectrum also had the NAc shift of α(2-3) and α(2-8) linked

Neu5Ac at δ 2.028. As the $^{1}$H-NMR spectrum was identical to that of authentic di-*N*-acetylneuraminyl lactose, it was characterized to be Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc.

**II-3-2-2. Assignment of $^{1}$H-NMR spectrum obtained from AC1-3.**

$^{1}$H-NMR spectrum (chemical shifts in Table 1) of the oligosaccharide in AC1-3 had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.223, 4.663 and 4.523, respectively. The spectrum (Fig. 5) also had the H-3 axial shifts of α(2-3) and α(2-8) linked Neu5Gc at δ 1.750; the signal intensity corresponded to two protons. It had the H-3 equatorial shifts of α(2-3) linked and α(2-8) linked Neu5Gc at δ 2.775 and 2.695, respectively, and NGc of α(2-3) and α(2-8) linked Neu5Gc at δ 4.117. As the other chemical shifts were rather similar to those of authentic di-N-acetylneuraminyl lactose, it was characterized to be Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc.

**II-3-2-3. Assignment of $^{1}$H-NMR spectrum obtained from AC1-4.**

$^{1}$H-NMR spectrum (chemical shifts in Table 1) had the shifts at 7.990 and 5.973, which arose from uracil, and the shifts at δ 5.963, 4.357, 4.295, 4.249 and 4.201, which were caused by ribose, showing the presence of uridine diphosphate. The spectrum (Fig. 6) had the H-1 shifts of α-GlcNAc and β(1-4) linked Gal at δ 5.515 and 4.436, respectively. H-3 axial and equatorial of α(2-6) linked Neu5Gc at δ 1.730 and 2.703, respectively and NGc of Neu5Gc at δ 4.112. The spectrum also had the NAc shift of GlcNAc at δ 2.095. As the spectrum was essentially similar to that of SC2-12 (Sasaki *et al*, 2016), which was separated from ovine colostrum, the saccharide in this fraction was characterized to be Neu5Gc(α2-6)Gal(β1-4)GlcNAcα1-UDP. This characterization was also supported by the MALDI-TOF/MS spectrum of this fraction. The MS (Fig. 14) spectrum had the ions at 1153.223, 1175.179, 1191.200, 1213.142 and 1229.139 which corresponded to $[M+2K-H]^{+}$, $[M+Na+2K-2H]^{+}$, $[M+3K-2H]^{+}$, $[M+Na+3K-3H]^{+}$ and $[M+4K-3H]^{+}$ respectively. These results are consistent with a molecular mass of 1076 as calculated from its chemical structure of Neu5Gc(α2-6)Gal(β1-4)GlcNAcα1-UDP.

**II-3-2-4. Assignment of $^{1}$H-NMR spectrum obtained from AC2-4.**

$^1$H-NMR spectrum of AC2-4 (chemical shifts in Table 2) had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.223, 4.663 and 4.529, respectively. The spectrum (Fig. 7) contained the H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac at δ 1.799 and 2.756, respectively, NAc shift of α(2-3) linked Neu5Ac at δ 2.030, and H-3 shift of β(1-4) linked Gal, which was substituted at OH-3, at δ 4.124. As this pattern was similar to those of authentic 3'SL, one saccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-4)Glc. The $^1$H-NMR spectrum had, in addition, the shifts at δ 7.955 and 5.977, which were caused by uracil of this fraction, and the other characteristic shifts at δ 5.955, 4.371, 4.284, 4.262 and 4.196, which were caused by ribose. The spectrum had the anomeric shift at δ 5.547 and the NAc shift at δ 2.081. These showed that this fraction contained HDP-hexodsamine. As the retention time of the fraction AC2-4 was same as that of authentic UDP-GlcNAc, one component in this fraction was tentatively identified to be UDP-GlcNAc.

**II-3-2-5. Assignment of $^1$H-NMR spectrum obtained from AC2-5.**

$^1$H-NMR spectrum of fraction AC2-5 (chemical shifts in Table 2) showed that this fraction contained more than two sialyl oligosaccharides. The spectrum (Fig. 8) had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.223, 4.664 and 4.531, respectively. The spectrum had the H-3 axial and equatorial shifts of α(2-3) linked Neu5Gc at δ 1.814 and 2.761, respectively, and NGc shift of α(2-3) linked Neu5Gc at δ 4.120. From these observations, one saccharide in this fraction was characterized to be Neu5Gc(α2-3)Gal(β1-4)Glc (AC2-5-1). The spectrum had another anomeric shifts of β-Glc and β(1-4) linked Gal at δ 4.669 and 4.428, respectively, and H-3 axial and equatorial shifts of α(2-6) linked Neu5Ac at δ 1.739 and 2.699, respectively, and NAc shift at 2.030. From these observations, another saccharide in this fraction was characterized to be Neu5Ac(α2-6)Gal(β1-4)Glc (AC2-5-2). The spectrum also had other anomeric shifts of α-GlcNAc, β-GlcNAc and β(1-4) linked Gal at δ 5.120, 40709 and 4.445, respectively, H-3 axial and equatorial shifts of α(2-6) linked Neu5Gc at δ 1.730 and 2.673, respectively, and NAc of reducing GlcNAc at δ 2.066; these showed the presence of Neu5Gc(α2-6)Gal(β1-4)GlcNAc (AC2-5-3). The spectrum, in addition, contained the down field shifts at δ 7.948, 5.985 and 5.972, showing the

presence of UDP-saccharide. As the retention time of AC2-5 during the HPLC was similar as that of UDP-Gal, this component was tentatively identified as UDP-Gal.

**II-3-2-6. Assignment of $^{1}$H-NMR spectrum obtained from AC2-6.**

$^{1}$H-NMR spectrum (chemical shifts in Table 2) of AC2-6 had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.227, 4.671 and 4.431, respectively. The spectrum (Fig. 9) had the H-3 axial and equatorial, and NGc shifts of α(2-6) linked Neu5Gc at δ 1.758, 2,716 and 4.120, respectively. From these assignments, it was concluded that the fraction contained Neu5Gc(α2-6)Gal(β1-4)Glc.

**II-3-2-7. Assignment of $^{1}$H-NMR spectrum obtained from AC3-3.**

$^{1}$H-NMR spectrum of AC3-3 (chemical shifts in Table 3 and Fig. 10) had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.224, 4.664 and 4.533, respectively, and H-3 axial, equatorial and NGc of α(2-3) linked Neu5Gc at δ 1.815, 2.761 and 4.123, respectively. From these observations, one saccharide in AC3-3 was characterized to be Neu5Gc(α2-3)Gal(β1-4)Glc. The spectrum, in addition, had the shifts at δ 7.946 and 5.984, which arose from uracil, and shifts at δ 5.964, 4.375, 4.365, 4.286, 4.254 and 4.216, which were caused by ribose, showing the presence of uridine diphosphate. The spectrum also had α-anomeric shifts at δ 5.631 and 5.601; these showed the presence of two UDP-hexoses. As the retention time of AC3-3 during the HPLC was different from that of UDP-Gal, it was concluded that these were not UDP-Gal.

**II-3-2-8. Assignment of $^{1}$H-NMR spectrum obtained from AC3-4.**

$^{1}$H-NMR spectrum (chemical shifts in Table 3) of AC3-4 had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.225, 4.670 and 4.430, respectively. The spectrum (Fig. 11) had the H-3 axial and equatorial, and NGc shifts of α(2-6) linked Neu5Gc at δ 1.763, 2.739 and 4.117, respectively. From these interpretations of the chemical shifts, the oligosaccharide in this fraction was characterized to be Neu5Gc(α2-6)Gal(β1-4)Glc. The spectrum, in addition, had the shifts at δ 7.953 and 5.981, which arose from uracil, the shifts at δ 5.965, 4.376, 4.365, 4.288, 4.258 and

4.211, which were caused by ribose and α-anomeric shift at δ 5.637. As the retention time of AC3-4 was same as that UDP-Gal, another component in this fraction was tentatively identified as UDP-Gal.

**II-3-2-9. Assignment of $^{1}$H-NMR spectrum obtained from AC4-4.**

$^{1}$H-NMR spectrum of AC4-4 (Fig. 12) had the shifts at δ 7.954 and 5.985, which arose from uracil, the shifts at δ 5.968, 4.376, 4.369, 4.292, 4.273 and 4.218, which were caused by ribose, and α-anomeric shift at δ 5.631; these showed the presence of UDP-hexose. As the retention time of AC4-4 was different from that of UDP-Gal during the HPLC, it was concluded that this was not UDP-Gal.

**II-3-2-10. Assignment of $^{1}$H-NMR spectrum obtained from AC4-5.**

$^{1}$H-NMR spectrum (Fig. 13) had the shifts at δ 8.114 and 5.938, which arose from uracil, and the shifts at δ 5.926, 4.355 and 4.213, which were caused by ribose. However, the α-anomeric shift was not found in the spectrum. From these these observations, it was assumed that the fraction contained a nucleotide.

**II-3-2-11. Assignment of $^{1}$H-NMR spectrum obtained from AC4-6.**

$^{1}$H-NMR spectrum had the shifts at δ 7.985 and 5.984, which arose from uracil, the shifts at δ 5.978, which was caused by ribose, and α-anomeric shift at δ 5.637. As the retention time of AC4-6 was similar to that of UDP-Gal during the HPLC, one component in this fraction was tentatively UDP-Gal. On the other hand, the spectrum had a lot of other chemical shifts, showing the presence of other components in this fraction.

**II-3-2-12. Unidentified fractions.**

Those of fractions were not characterized due to unclear $^{1}$H-NMR spectrums obtained in this study: AC1-1, AC1-5, AC1-6, AC1-7, AC1-8, AC1-9, AC1-10, AC2-1, AC2-2, AC2-3 AC3-1, AC3-2, AC4-1, AC4-2, AC4-3 and AC4-7.

**II-4. Discussion**

In this study, several chemical structures of acidic oligosaccharides including sugar nucleotides were characterized as shown in Table 4. In the addax colostrum, sialyllactoses and sialyl *N*-acetyllactosamines were found as follows: Neu5Ac(α2-3)Gal(β1-4)Glc, Neu5Ac(α2-6)Gal(β1-4)Glc, Neu5Ac(α2-6)Gal(β1-4)GlcNAc, Neu5Gc(α2-3)Gal(β1-4)Glc, Neu5Gc(α2-6)Gal(β1-4)Glc and Neu5Gc(α2-6)Gal(β1-4)GlcNAc. According to previous studies, these oligosaccharides were found at different proportion in mature milk or colostrum of cows, goats and sheep (Albrecht *et al,* 2014; Urashima *et al,* 2016). The sialic acid (Neu5Ac) and glycolic acid (Neu5Gc) ratio in these sialyllactoses and sialyl *N*-acetyllactosamines was almost equal in addax colostrum. Neu5Gc containing oligosaccharides were as a major while Neu5Ac containing oligosaccharides were as a minor in ovine colostrum (Nakamura *et al,* 1998). Sasaki *et al* (2016) reported that Neu5Gc containing oligosaccharides were present while Neu5Ac containing oligosaccharides were absent in ovine colostrum of the Corydale breed. Previous studies on bovine milk oligosaccharides indicated that Neu5Ac containing oligosaccharides in the colostrum predominate over those containing Neu5Gc (Albrecht *et al,* 2014; Urashima *et al,* 2016). It has been also described that the ratio of Neu5Ac/Neu5Gc in sialyl oligosaccharides varies depending on the species as follows; 97:3 in the cow, 37:64 in the goat and 6:94 in sheep (Albrecht *et al,* 2014). The predominance of oligosaccharides containing Neu5Ac/Neu5Gc(α2-3) over those containing Neu5Ac/Neu5Gc(α2-6) was observed in the addax colostrum; this finding was corresponding to previous findings for cows and sheep, but different from goats (Urashima *et al,* 2016).

Previous studies indicated that milk oligosaccharides containing Neu5Ac and Neu5Gc, disialyllactoses Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc and Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc were identified in bovine colostrum and mature milk (Marino *et al,* 2011; Albrecht *et al,* 2014), sheep (Albrecht *et al,* 2014) and goat (Albrecht *et al,* 2014), while Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc was found in bovine colostrum (Marino *et al,* 2011; Albrecht *et al,* 2014). Other disilayllactoses such as Neu5Gc(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc and Neu5Ac(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc have been identified in milk and colostrum of cows, goats and sheep

(Albrecht *et al,* 2014; Marino *et al,* 2011). Although these two disialyllactoses were not present in the addax colostrum, they may exists a minor component in addax colostrum, even though this could not been detected in this study.

In addition, a nucleotide connected to sialyl *N*-acetyllactosamine, Neu5Gc(α2-6)Gal(β1-4)GlcNAc-α1-UDP, was identified in the addax colostrum. This oligosaccharide nucleotide was previously identified in ovine colostrum (Sasaki *et al,* 2016). On the other hand, Neu5Gc(α2-3)Gal(β1-4)GlcNAc-α1-UDP which was found by Sasaki *et al* (2016) in ovine colostrum, was not identified. It is possible that this nucleotide structure occurs as a minor component in addax colostrum. Because $^{1}$H-NMR spectrum showed the weak shift and intensities of UDP-Neu5Gc-LacNAc and Neu5Gc-lactose. Other nucleotides of the oligosaccharide, such as Gal(β1-4)GlcNAc-α1-UDP and Fuc(α1-2)Gal(β1-4)GlcNAc-α1-UDP were found in reindeer (Taufik *et al,* 2014), pig (Kobata & Suzuki, 1965) and human (Kobata, 1963; 1966) mature milk and colostrum. Previous study indicated that Sia(α2-3(6))Gal(β1-4) GlcNAc-α1-UDP and Sia(α2-3(6))Gal(β1-6)GlcNAc-α1-UDP were identified in goat colostrum (Jourdian *et al,* 1961), even though they were not identified in caprine colostrum in our previous study (Urashima, 2017). Colostrums of sheep and addax were found to contain Neu5Gc(α2-6)Gal(β1-4)GlcNAcα1-UDP in the previous and this experiments, but it was absent in goat's milk. Among the closely related species of Bovidae such as cows, water buffalo, buffalo as well as some antelopinae, it is interesting to know that which species mature milk and colostrum contain UDP sialyl *N*-acetyllactosamine or UDP *N*-acetyllactosamine.

Sugar nucleotides such as UDP-Gal, UDP-GlcNAc, CMP-Neu5Ac, GDP-Fuc and so on, mostly function as donors for glycosyltransferases that transfer monosaccharides to acceptor glycoconjugates within the Golgi apparatus of mamary epithelial cells (Sasaki *et al*, 2016). Theoretically, UDP oligosaccharides mentioned in this study could be utilized as donors for glycosyltransferase enzymes that transfer *N*-acetyllactosamine (LacNAc), fucosyl LacNAc (Fuc(α1-2)Gal(β1-4)GlcNAc) or sialyl LacNAc to suitable acceptors. Up to date, glycosyltransferases never been detected in any biological source, especially in the milk and

colostrum. The UDP oligosaccharides in milk and colostrum might have other bio-functional benefits for the neonates rather than as donors for glycolsyltransferases.

As previously suggested by Urashima *et al* (2011) and Bode (2012), the oligosaccharides in mature milk and colostrum act as prebiotics that stimulate the growth of bifidobacteria in the infant colon, as decoy receptors that inhibit the attachment of pathogenic microorganisms to the colonic epithelium, and as modulating factors for the development of colon epithelial cells. The modulating effect of bovine milk oligosaccharides isolated from cheese whey on infant colonic microflora was observed by Charbonneau *et al* (2016). Probably, the UDP-oligosaccharides in mature milk and colostrum have similar functions, as UDP sialyl *N*-acetyllactosamine has homologous sugar moiety as oligosaccharides in bovine milk. In the future, *in vitro* studies should be conducted to discover the possible function of milk or colostrum oligosaccharides with UDP-oligosaccharides as well as with colonic epithelial cells, some bifidobacterial strains and pathogenic bacterial strains.

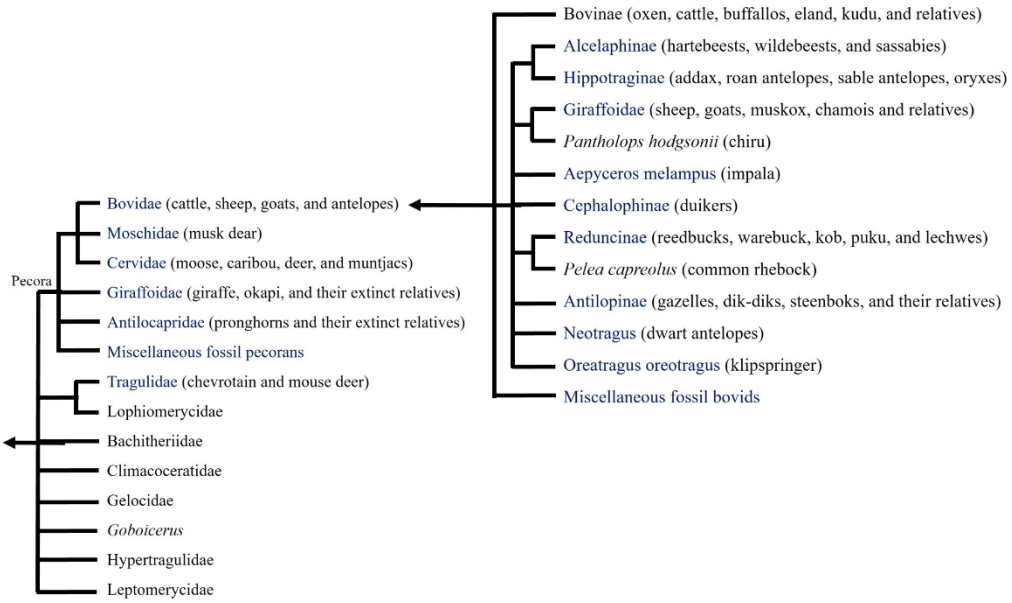


Fig 1. Phylogenetic tree of Bovidae family (adopted from http://tolweb.org/Ruminantia/16001).

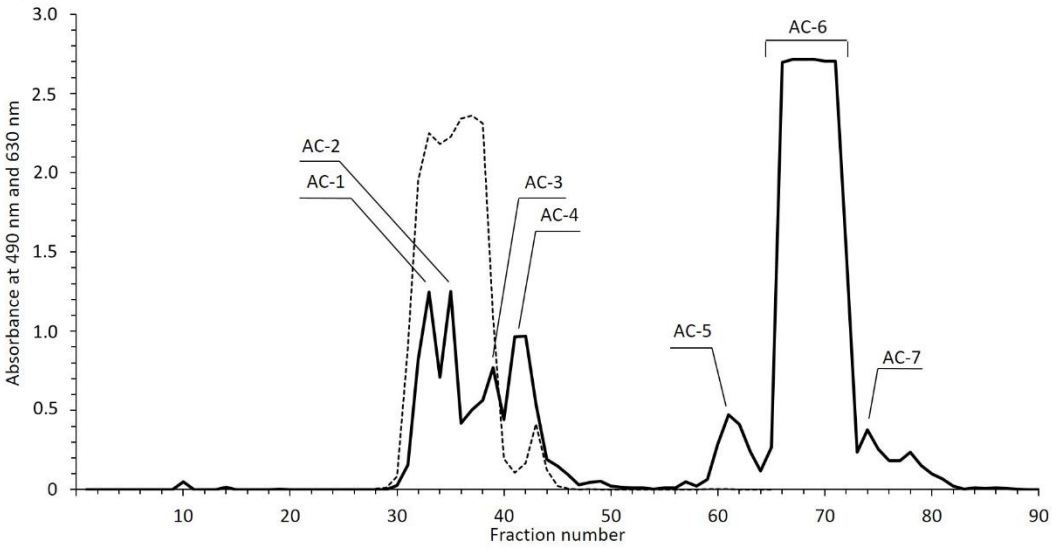


Fig. 2. Gel chromatogram of the carbohydrate fraction extracted from addax milk using a BioGel P-2 column (2.5 ×100 cm). Elution was done with distilled water at a flow rate of 15ml/h and fractions of 5.0 ml were collected. Each fraction was monitored for hexose by the phenol-$H_2SO_4$ method at 490 nm (solid line) and for sialic acid by periodate-resorcinol at 630 nm (dotted line).

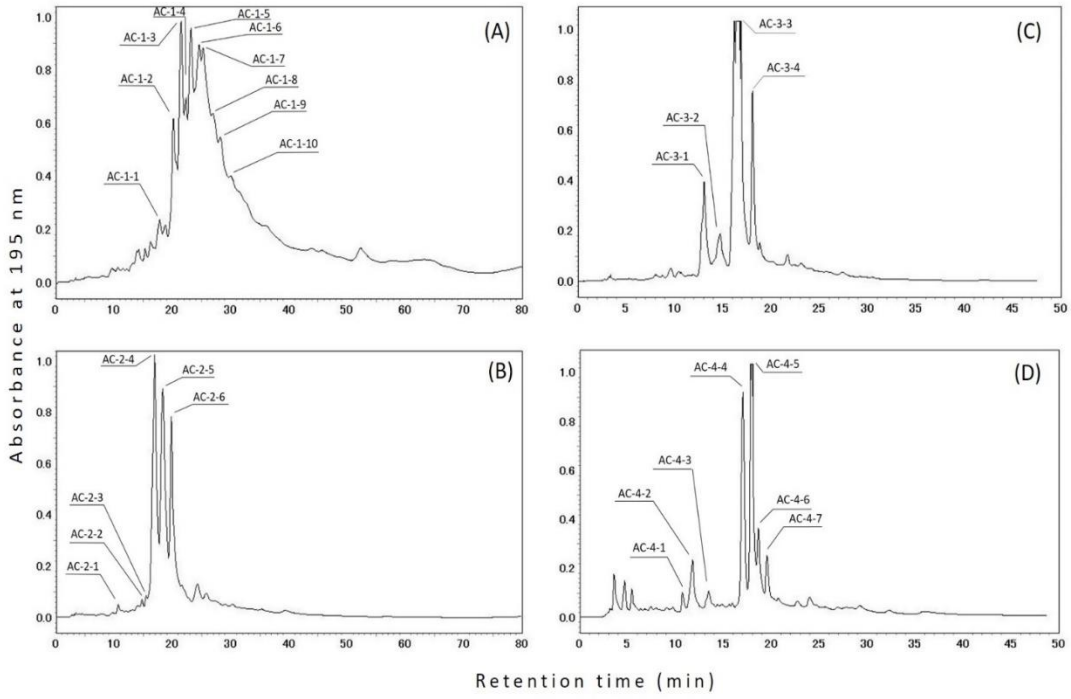


Fig. 3. High performance liquid chromatograms using a TSK-gel Amide-80 column of (A) fraction AC-1, (B) fraction AC-2, (C) fraction AC-3 and (D) fraction AC-4, separated from the carbohydrate fraction of addax colostrum by gel chromatography.

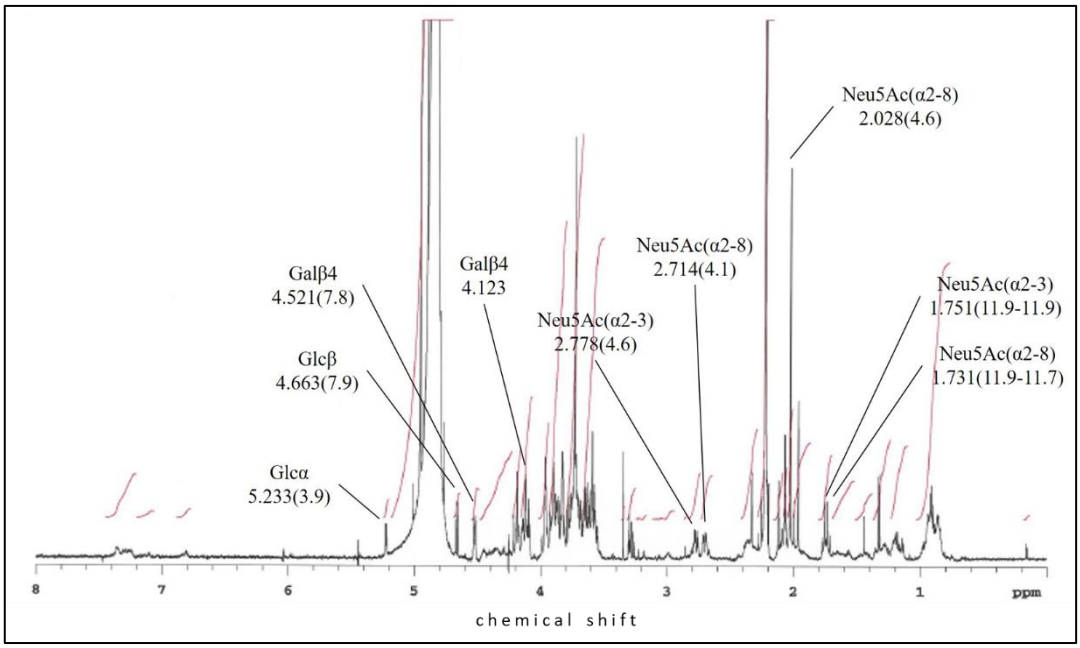


Fig. 4. The $^{1}$H-NMR spectrum of AC1-2 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). Glc, glucose; Gal, galactose; Neu5Ac, *N*-acetylneuraminic acid.

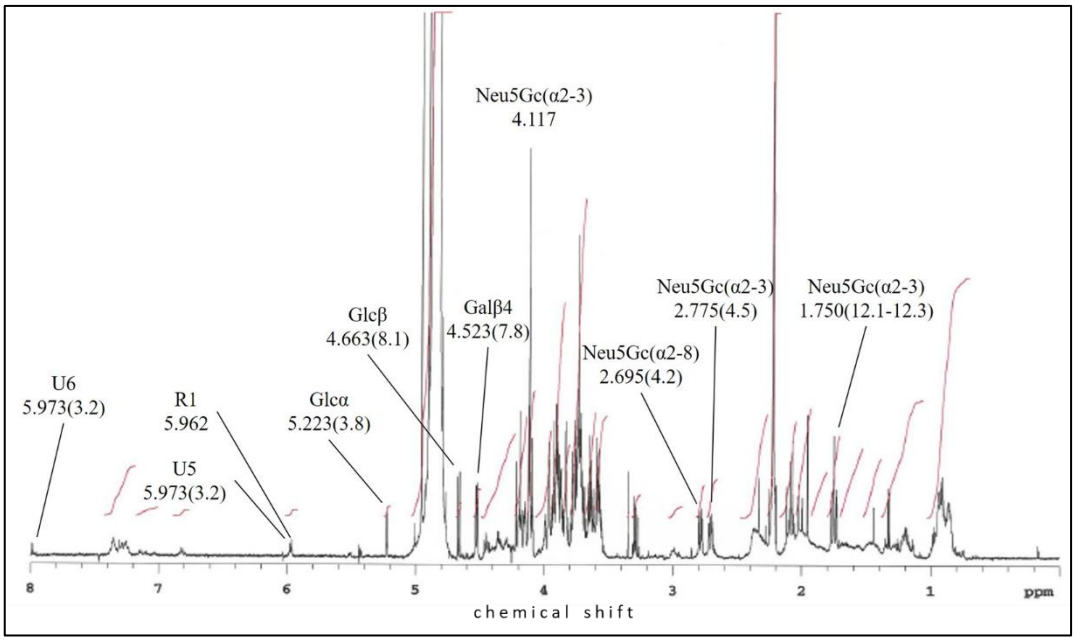


Fig. 5. The $^{1}$H-NMR spectrum of AC1-3 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Glc, glucose; Gal, galactose; Neu5Gc, *N*-glycolylneuraminic acid.

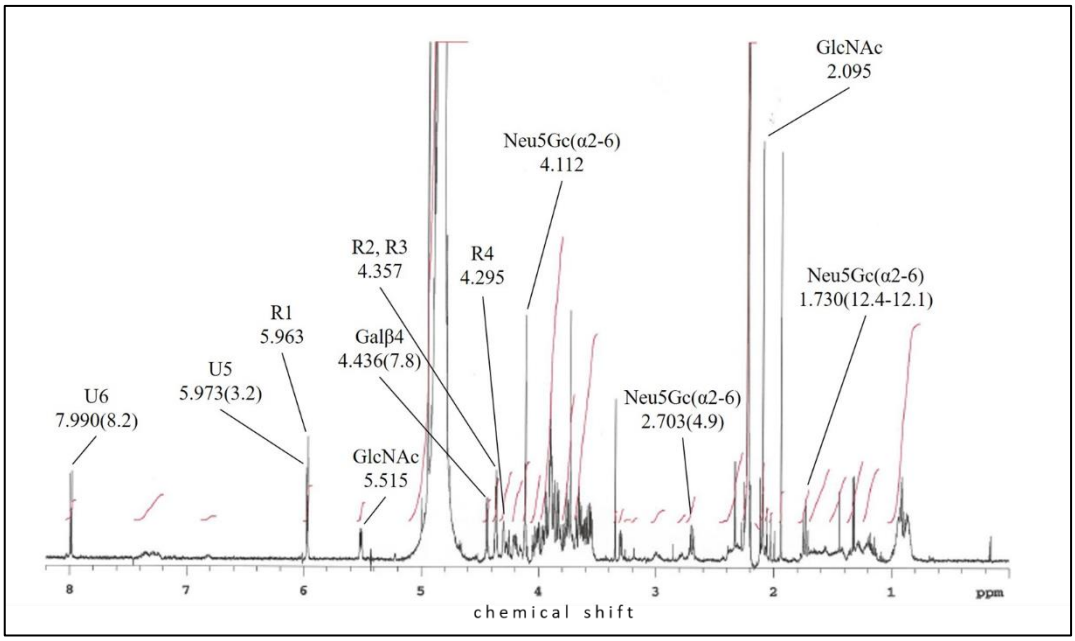


Fig. 6. The $^{1}$H-NMR spectrum of AC1-4 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Gal, galactose; GlcNAc, *N*-acetylglucosamie; Neu5Ac, *N*-acetylneuraminic acid.

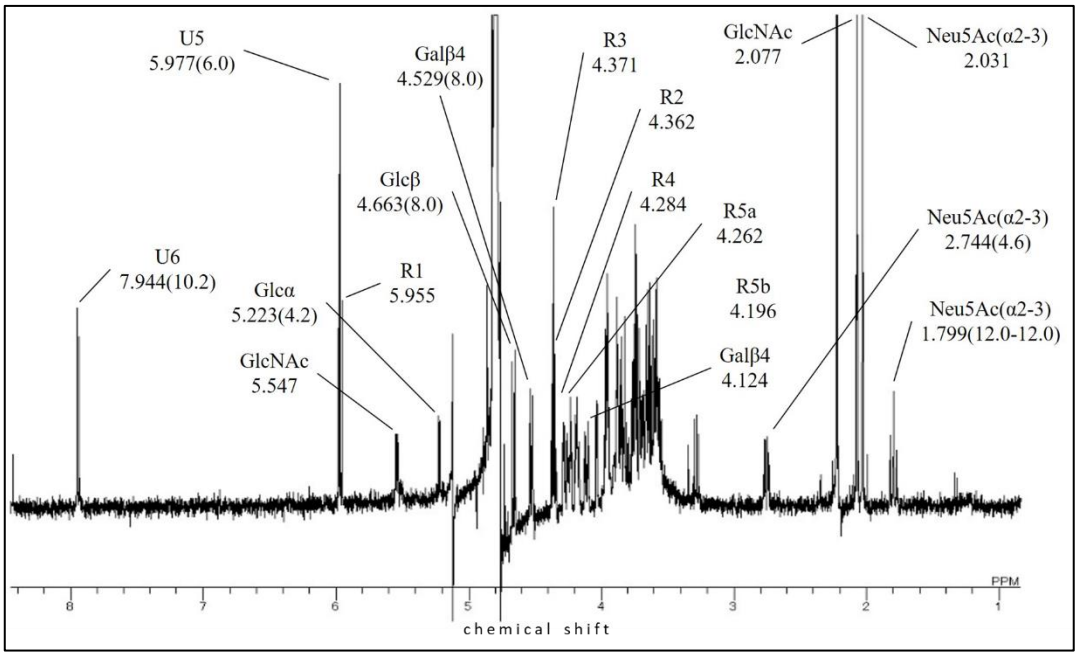


Fig. 7. The $^{1}$H-NMR spectrum of AC2-4 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamie; Neu5Ac, *N*-acetylneuraminic acid.

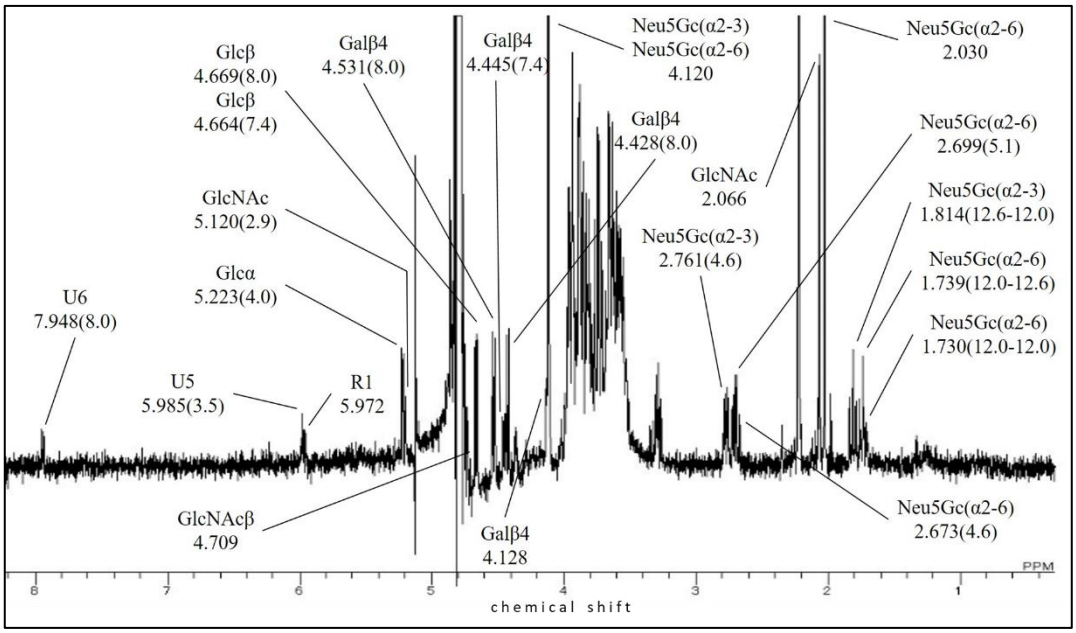


Fig. 8. The $^{1}$H-NMR spectrum of AC2-5 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamie; Neu5Gc, *N*-glycolylneuraminic acid.

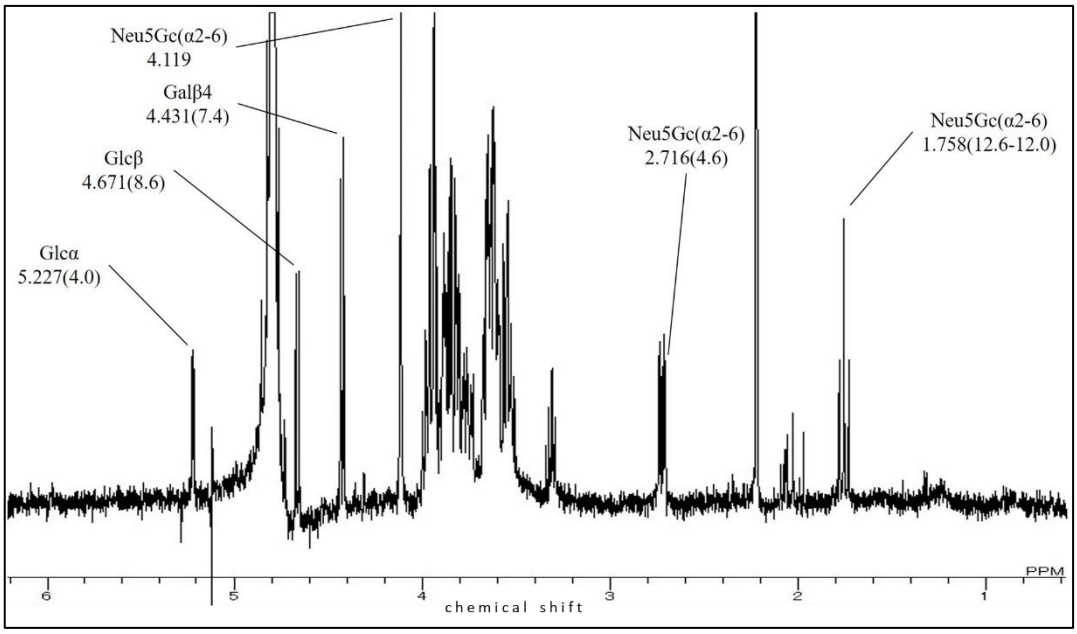


Fig. 9. The NMR spectrum of AC2-6 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). Glc, glucose; Gal, galactose; Neu5Ac, *N*-acetylneuraminic acid.

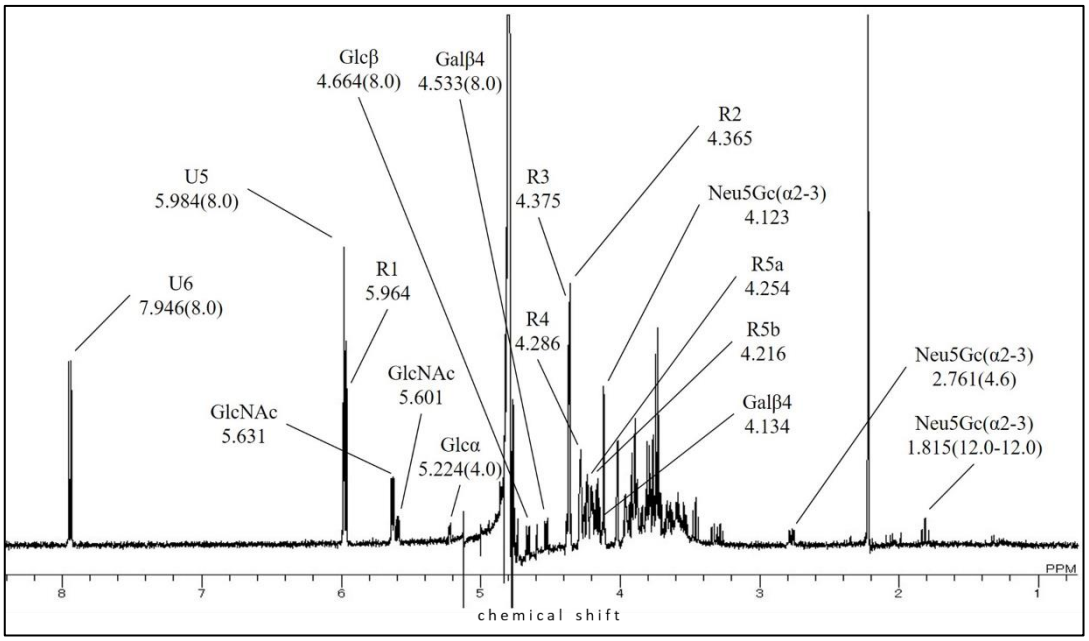


Fig. 10. The NMR spectrum of AC3-3 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamie ; Neu5Gc, *N*-glycolylneuraminic acid.

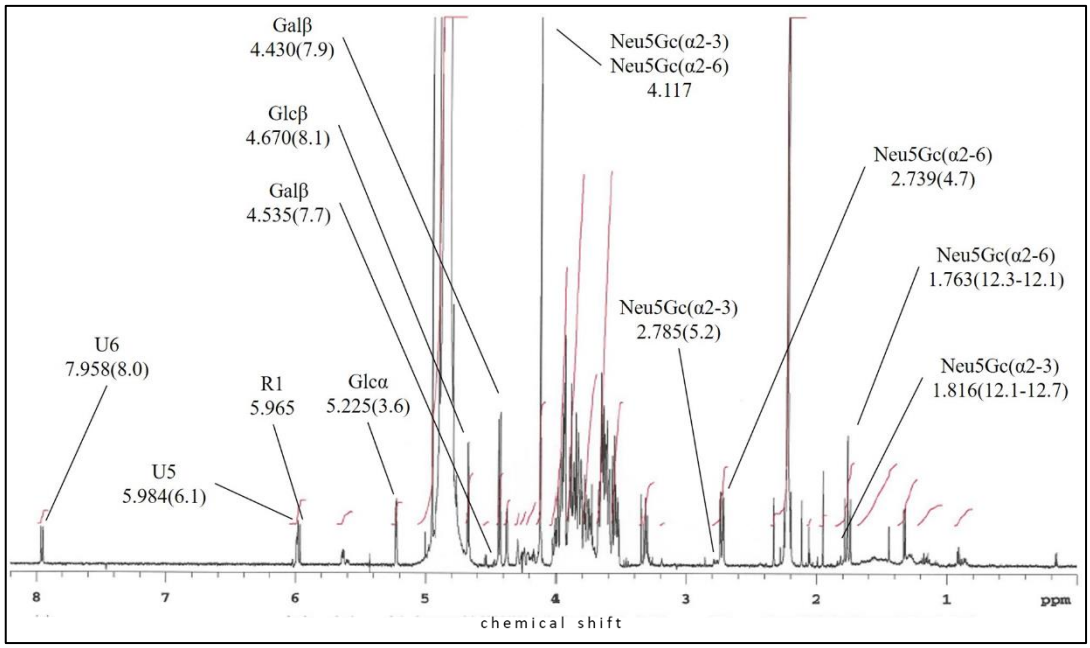


Fig. 11. The NMR spectrum of AC3-4 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Glc, glucose; Gal, galactose; Neu5Gc, *N*-glycolylneuraminic acid.

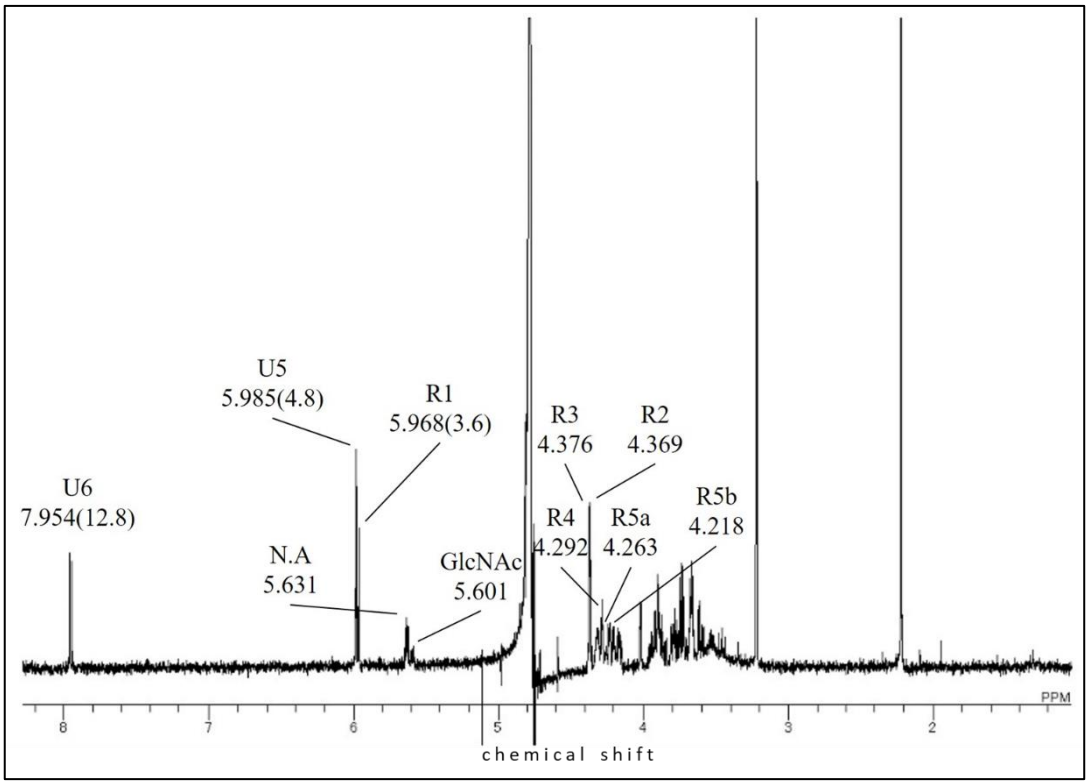


Fig. 12. The NMR spectrum of AC4-4 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; Rib, ribose; GlcNAc, *N*-acetylglucosamie; N.A, not assigned.

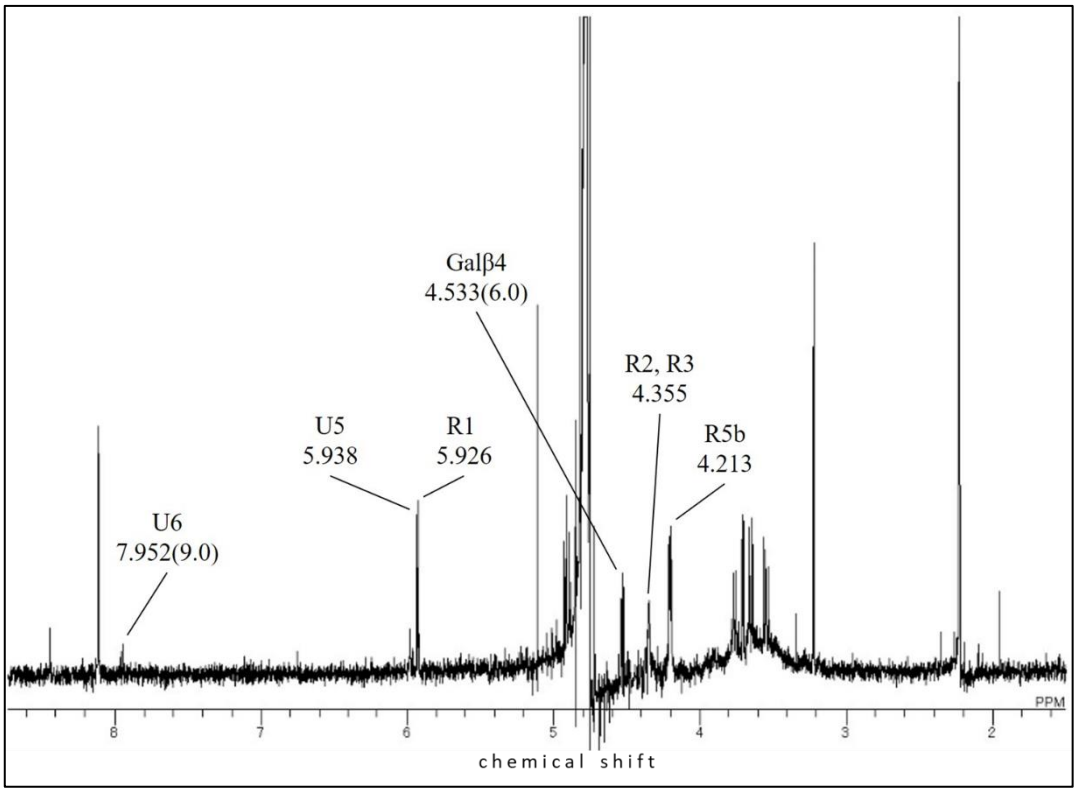


Fig. 13. The $^{1}$H-NMR spectrum of AC4-5 from addax colostrum. The spectrum was obtained in $D_2O$ at 600 MHz with a Varian INOVA 600 spectrometer operated at 293.1 K (Varian, Palo Alto, CA, USA). U, uracil; R, ribose; Gal, galactose.

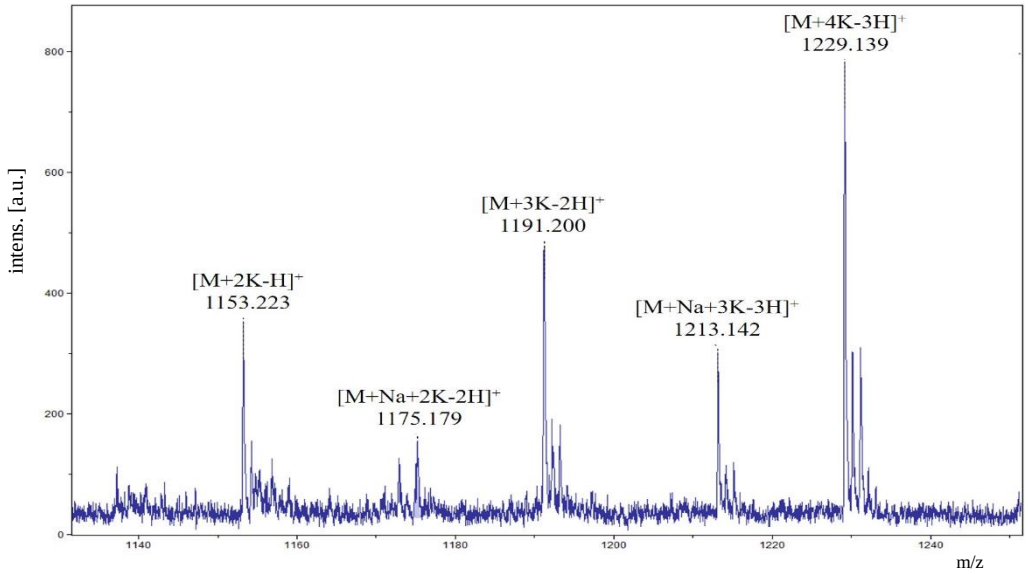


Fig. 14. Matrix-assisted, laser-desorption time-of-light (MALDI-TOF) mass spectra of the UDP-sialyl *N*-lactosamines on the fraction AC1-4 isolated from addax colostrum carbohydrate by HPLC. The spectra were performed using an Autoflex II TOF/TOF mass spectrometer (Bruker Faltonics, Bremen, Germany). The sample solution was mixed on a target plate (MTP 374 ground steel TF, Bruker), with an equal volume of 10 mg/mL SDHB (Bruker Daltonics) saturated in distilled water. Mass spectra were obtained using a reflector positive ion mode optimized to mass range of 0-3 kDa. Peptide calibration standard II (Bruker Daltonics) was used for external calibration of the mass spectrometer.

**Table 1.** Assignment of proton nuclear magnetic resonance spectroscopy ([1]H-NMR) chemical shifts of fraction AC 1-2 to AC 1-4 separated from addax colostrum.

| Reporter group[1] | Residue[2] | Chemical shifts, δ(coupling constants, Hz) | | |
|---|---|---|---|---|
| | | AC1-2 | AC1-3 | AC1-4 |
| H-1 | Glcα | 5.223(3.9) | 5.222(3.8) | - |
| | Glcβ | 4.663(7.9) | 4.663(8.1) | - |
| | Gal(β1–4) | 4.521(7.8) | 4.523(7.8) | 4.436(7.8) |
| | GlcNAcα | - | - | 5.515 |
| H-3ax | Neu5Ac(α2-3) | 1.751(11.9[a]-11.9[b]) | - | - |
| | Neu5Gc(α2-3) | - | 1.750(12.1[a]-12.3[b]) | - |
| | Neu5Gc(α2-6) | - | - | 1.730(12.4[a]-12.1[b]) |
| | Neu5Ac(α2-8) | 1.731(11.9[a]-11.7[b]) | - | - |
| | Neu5Gc(α2-8) | - | 1.750(12.1[a]-12.3[b]) | - |
| H-3eq | Neu5Ac(α2-3) | 2.778(4.6[c]) | - | - |
| | Neu5Gc(α2-3) | - | 2.775(4.5[c]) | - |
| | Neu5Gc(α2-6) | - | - | 2.703(4.9[c]) |
| | Neu5Ac(α2-8) | 2.714(4.1[c]) | - | - |
| | Neu5Gc(α2-8) | - | 2.695(4.2[c]) | - |
| H-3 | Gal(β1–4) | 4.123 | 4.125 | 4.116 |
| NAc | Neu5Ac(α2-3) | 2.028 | - | - |
| | Neu5Ac(α2-8) | 2.028 | - | - |
| | GlcNAcα | - | - | 2.095 |
| NGc | Neu5Gc(α2-3) | - | 4.117 | - |
| | Neu5Gc(α2-6) | - | - | 4.112 |
| | Neu5Gc(α2-8) | - | 4.117 | - |

[a]$J_{3ax, 4}$; [b]$J_{3ax, 3eq}$; [c]$J_{3eq, 4}$, where $J_{x, y}$ indicates the coupling constant of H-x and H-y.
[1]H-3ax, H-3 axial; H-3eq, H-3 equatorial; NAc, *N*-acetyl; NGc, *N*-glycolyl.
[2]Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid.

**Table 2.** Assignment of proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) chemical shifts of fraction AC 2-2 to AC 2-6 separated from addax colostrum.

| Reporter group[1] | Residue[2] | Chemical shifts, δ(coupling constants, Hz) | | | | |
|---|---|---|---|---|---|---|
| | | AC2-4 | AC2-5-1 | AC2-5-2 | AC2-5-3 | AC2-6 |
| H-1 | Glcα | 5.223(4.0) | - | 5.223(4.0) | 5.223(4.0) | 5.227(4.0) |
| | Glcβ | 4.663(8.0) | - | 4.664(7.4) | 4.669(8.0) | 4.671(8.6) |
| | GlcNAcα | - | 5.120(2.9) | - | - | - |
| | GlcNAcβ | - | 4.709 | - | - | - |
| | Gal(β1−4) | 4.523(7.4) | 4.445(7.4) | 4.531(8.0) | 4.428(8.0) | 4.431(7.4) |
| H-3ax | Neu5Ac(α2-3) | 1.799($12.0^b$-$12.0^c$) | - | - | - | - |
| | Neu5Gc(α2-3) | - | - | 1.814($12.6^a$-$12.0^b$) | - | - |
| | Neu5Ac(α2-6) | - | - | - | 1.739($12.0^a$-$12.6^b$) | - |
| | Neu5Gc(α2-6) | - | 1.730($12.0^a$-$12.6^b$) | - | - | 1.758($12.6^a$-$12.0^b$) |
| H-3eq | Neu5Ac(α2-3) | 2.744($4.6^c$) | - | - | - | - |
| | Neu5Gc(α2-3) | - | - | 2.761($4.6^c$) | - | - |
| | Neu5Ac(α2-6) | - | - | - | 2.699($5.1^c$) | - |
| | Neu5Gc(α2-6) | - | 2.673($4.6^c$) | - | - | 1.758($12.6^a$-$12.0^b$) |
| H-3 | Gal(β1−4) | 4.124 | - | 4.128 | - | - |
| NAc | Neu5Ac(α2-3) | 2.030 | - | - | - | - |
| | Neu5Ac(α2-6) | - | - | - | 2.030 | - |
| | GlcNAc | - | 2.066 | - | - | - |
| NGc | Neu5Gc(α2-3) | - | - | 4.120 | - | - |
| | Neu5Gc(α2-6) | - | 4.120 | - | - | 4.120 |

[a]$J_{3ax,\ 4}$; [b]$J_{3ax,\ 3eq}$; [c]$J_{3eq,\ 4}$, where $J_{x,\ y}$ indicates the coupling constant of H-x and H-y.
[1]H-3ax, H-3 axial; H-3eq, H-3 equatorial; NAc, *N*-acetyl; NGc, *N*-glycolyl.
[2]Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid.

**Table 3.** Assignment of proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) chemical shifts of fraction AC 3-3 and AC 3-4 separated from addax colostrum.

| Reporter group[1] | Residue[2] | Chemical shifts, δ(coupling constants, Hz) | |
|---|---|---|---|
| | | AC3-3 | AC3-4 |
| H-1 | Glcα | 5.224(4.0) | 5.225(3.6) |
| | Glcβ | 4.664(8.0) | 4.670(8.0) |
| | Gal(β1−4) | 4.533(8.0) | 4.430(8.0) |
| H-3ax | Neu5Gc(α2-3) | 1.815($12.0^a$-$12.0^b$) | - |
| | Neu5Gc(α2-6) | - | 1.763($12.3^a$-$12.1^b$) |
| H-3eq | Neu5Gc(α2-3) | 2.761($4.6^c$) | - |
| | Neu5Gc(α2-6) | - | 2.739($4.7^c$) |
| H-3 | Gal(β1−4) | 4.134 | |
| NGc | Neu5Gc(α2-3) | 4.123 | - |
| | Neu5Gc(α2-6) | - | 4.117 |

[a] $J_{3ax,\ 4}$; [b] $J_{3ax,\ 3eq}$; [c] $J_{3eq,\ 4}$, where $J_{x,\ y}$ indicates the coupling constant of H-x and H-y.
[1]H-3ax, H-3 axial; H-3eq, H-3 equatorial; NAc, *N*-acetyl; NGc, *N*-glycolyl.
[2]Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid.

**Table 4.** Chemical structures of sialyllactose, sialyl *N*-acetyllactosamine, UDP-sialyl *N*-acetyllactosamine and other sugar nucleotides separated from addax colostrum.

| Fraction | Structure |
|---|---|
| AC-1-2 | Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc |
| AC-1-3 | Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc |
| AC-1-4 | Neu5Gc(α2-6)Gal(β1-4)GlcNAc-UDP |
| AC-2-4 | Neu5Ac(α2-3)Gal(β1-4)Glc |
| | UDP-GlcNAc |
| AC-2-5 | Neu5Gc(α2-6)Gal(β1-4)GlcNAc |
| | Neu5Gc(α2-3)Gal(β1-4)Glc |
| | Neu5Ac(α2-6)Gal(β1-4)Glc |
| | UDP-Gal |
| AC-2-6 | Neu5Gc(α2-6)Gal(β1-4)Glc |
| AC-3-3 | Neu5Gc(α2-3)Gal(β1-4)Glc |
| | UDP-hexose |
| AC-3-4 | Neu5Gc(α2-6)Gal(β1-4)GlcNAc |
| | UDP-Gal |
| AC-4-4 | UDP-hexose |
| AC-4-5 | Nucleotide |
| AC-4-6 | UDP-Gal |

Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc, di-*N*-acetylneuraminic acid; Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc, di-*N*-glycolylneuraminic acid; Neu5Gc(α2-6)Gal(β1-4)GlcNAc-UDP, uridine 5'-diphospho-N-acetyllactosamine; Neu5Gc(α2-6)Gal(β1-4)GlcNAc, 6'-glycolyllactosamine; Neu5Ac(α2-3)Gal(β1-4)Glc, 3'-sialyllactose; Neu5Gc(α2-3)Gal(β1-4)Glc, 3'-glycolyllactose; Neu5Ac(α2-6)Gal(β1-4)Glc, 6'-sialyllactose; Neu5Gc(α2-6)Gal(β1-4)Glc, 6'-glycolyllactose; UDP-GlcNAc, uridine 5'-diphospho-N-acetyl-D-glucosamine; UDP-hexose, uridine 5'-diphospho-N-acetyl-D-hexose; and UDP-Gal, uridine 5'-diphospho-N-acetyl-D-galactose.

# Chapter III

## Isolation and identification of peptides in the whey of Mongolian fermented camel's and mare's milks

### III-1. Introduction

Indigenous foods are very important sources of functional substances since they comprised with many unknown components. Moreover, traditional Mongolian dairy products are very likely to have several health beneficial aspects for human as described in Chapter I. Fermented camel's and mare's milks are respected by Mongolian nomads to have some health beneficial activities. For example, the fermented camel's milk has been used to treat watery swollen during the pregnancy period and as body strengthening for aged people during the seasonal changes in Mongolia (Dubach *et al*, 2007) while fermented mare's milk have positive impact to enhance the immune system and for treatment of liver, lung and heart disease (Wang *et al*, 2008; Yuan *et al*, 2006).

Whereas certain amount of peptides apparently exist in milk, most of bioactive peptides are encrypted in milk proteins in inactive form, and those peptides are released by the action of proteolytic enzymes during either fermentation process of dairy foods or digestion in the gastrointestinal tract of consumers (Meisel & Bockelmann, 1999; Korhonen & Pihlanto, 2006). Milk-derived bioactive peptides are of particular interest in food science and nutrition because they show important physiological and biological functions such as opioid-like (Chang *et al*, 1981), immunomodulating (Fiat *et al*, 1993), antibacterial (Bellamy *et al*, 1992), and antihypertensive activities (Hata *et al*, 1996), and the ability to enhance calcium absorption (Sato *et al*, 1986). From this point of view, a couple of comprehensive studies on dromedary camel's milk and its fermented product have been performed (Alhaider *et al*, 2013; Moslehishad *et al*, 2013). To date, however, there is little information available on peptides in fermented camel's and mare's milk products that are traditionally produced.

In this study, several peptides were isolated from fermented Mongolian camel's and mare's milks using an ultrafiltration device, whose nominal molecular weight limit (NMWL) was 3,000, and reverse phase high performance liquid chromatography (RP-HPLC). Amino acid sequences of the purified peptides were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with de novo sequencing.

## III-2. Materials and methods

### III-2-1. Reagents

Centriprep YM-3 filter device was from Millipore (Billerica, USA). Water was purified using Millipore Direct Quv3 Water Purification System (Millipore, Bedford, USA). Acetonitrile and TFA were purchased from Sigma in Japan. Buffer solutions for RP-HPLC were filtered through 0.2 micron membrane filter from Millipore prior to use. The peptide calibration standard II, 1-3 kDa, used for calibration of the matrix-assisted laser desorption ionization time of flight tandem mass spectrometer (MALDI-TOF MS/MS) and MALDI target plate was from Bruker Daltonik GmbH (Bremen, Germany). The matrix α-cyano-4-hydroxycinnamic acid (CHCA) used for MALDI-TOF MS/MS analysis was purchased from Bruker Daltonik GmbH. The HPLC system was a JASCO (Tokyo, Japan) and equipped with a PU-2089 pump, UV 2075 detector, MX 2080-32 dynamic mixer, and a CO-8020 column oven. TSK gel ODS-80Ts (ϕ 0.46 cm × 25 cm) column was from Tosoh. All other chemicals were analytical grade.

### III-2-2. Fermented milk samples

Bactrian camel's fermented milks were collected about 500 mL each from 3 individual herders: two herders near to Hamriin hiid, Ulaanbadrakh soum, Dornogovi aimag and one herder in Dalanzadgad soum, Umnugovi aimag in Mongolia on October, 2016. Mare's fermented milks were also collected 3 herders or private farms in Adaatsag soum, Dundgovi aimag, Mongolia and approximate volume of each samples were 500 mL. All the samples were stored in cooling boxes during transport from the farms to the State Central Veterinary Laboratory in Ulaanbaatar and kept at -30°C until delivery to Japan.

### III-2-3. Peptide fractionation

Milk samples (100 mL) were defatted by centrifugation at 500 x *g* for 10 min at 20°C. The fat and cells were removed and then supernatant as a whey was centrifuged again at the same condition to remove residual cream. Residual caseins and insoluble compounds were removed by centrifugation at 39800 x *g* for 60 min at 4 °C. The supernatant, namely acid whey, was subjected to ultrafiltration at 4°C using Centriprep YM-3 (NMWL 3,000). Membrane permeate was lyophilized and dissolved in 0.1% of TFA at 80 mg/mL concentration. A 100-μL aliquot of membrane permeate was injected into ODS-80Ts column (ϕ 0.46 × 25 cm), pre-equilibrated with 0.1% TFA and connected to HPLC system, which is equipped with PU-2089 pump, UV 2075 detector, MX 2080-32 dynamic mixer, and CO-8020 column oven (Jasco Co.,Tokyo, Japan). After 5 min static flow with 100% solvent A (0.1% TFA in water), elution was performed by a linear gradient from 0 to 50% solvent B (0.1% TFA in ACN) for 90 min at a flow rate of 1.0 mL/min and at 30°C. The eluent was monitored by an ultraviolet detector at the wavelength of 214 nm. Eluted peptide fractions were collected manually, concentrated by rotary and vacuum evaporation, and lyophilized. The lyophilized peptides were dissolved in 50 μL of 0.1% TFA and stored at -20°C until used.

### III-2-4. Mass spectrometry

A peptide solution in 0.1% TFA desalted using ZipTip C18 pipette tips (Millipore, Bedford, USA) according to the manufacturer's instructions. A 1.0-μL aliquot of the desalted peptide solution and an equal volume of 10 mg/mL of α-CHCA saturated in 0.1% TFA/ACN (2:1, v/v) were mixed, and 1.0 μL of the mixture was loaded on a target plate (MTP 384 target plate ground steel TF, Bruker). After the solvent dried, the target plate was mounted in AutoflexII TOF/TOF mass spectrometer (Bruker). Mass spectra were obtained using the pre-installed method, RP_1-3kDa (a reflector positive ion mode optimized to the mass range of 1-3 kDa). Peptide calibration standard II was used as an external mass calibrant. The acquired spectra were statistically analyzed using flexAnalysis 2.0 software (Bruker). The peptide mass list was searched using Biotool 3.0 interface (Bruker) connected to the Mascot search engine. Amino acid sequences of the peptides

were estimated by fragmentation analysis of MALDI-generated ions using a technique of TOF/TOF MS (MS/MS). Homology search was done using a database at the Web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

### III-3. Results

#### III-3-1. Fermented milk peptide profiles on RP-HPLC

Whey fractions which is lower than <3 kDa, prepared from fermented camel's and mare's milks were separated by RP-HPLC. Fermented camel's milk (Fig. 15) and mare's milk (Fig. 16) showed distinct chromatograms on RP-HPLC. The number of major peaks in fermented camel's milk was fewer than mare's milk. An HPLC profile of fermented mare's milk was significantly different compare to fermented camel's milk (Fig. 17). In general, there were six major (labeled by red-headed arrow in Fig. 15) and many other minor peaks in each samples of fermented camel's milk (C1, C2 and C3 in Fig. 15). On the other hand, major peaks (labeled by red-headed arrow in Fig. 16) in the fermented mare's milk were ranged from 11 to 22 (11 peaks for H3, 15 peaks for H2 and 22 peaks for H1) whereas plenty of minor peaks observed in each of H1, H2 and H3 (Fig. 16). No peak appeared in all the chromatograms after 75 min to the end of elution. Based on these information, C3 and H1 samples were selected for further analysis to fractionate and identify peptides. In total, 33 peaks, which of 11 peaks from C3 (Fig. 17) and 22 peaks from H1 (Fig. 17) were manually collected and analyzed by mass spectrometry.

#### III-3-2. Peptide identification

Identification of peptides in the 11 peaks from fermented camel's milk was summarized in Table 5. In the fermented camel's milk, C3-3 was consisted a mixture of [RHQNQNPK] (Figs. 20.1A and B), lactophorin fragment and two unknown peptides with sequence of HLLQPF and NLRLPV. Also, lactophorin derived peptide [RRHQNQNPK] (Figs. 20.2A and B) was identified in the C3-4. In the C3-5, $\alpha_{s1}$-casein fragments, [DTRNEPTEDH] (Figs. 20.3A and B) and [TRNEPTEDH] (Fig. 20.3C) were determined. C3-6 and C3-7 were assigned to be κ-casein fragment, [RPRPRPS] (Figs. 20.4A, B and C). C3-8 was revealed as a mixture of unknown peptide

and β-casein peptides, [HPVPQP] (Figs. 20.5A and B) and [PDPVR] (Fig. 20.5C). C3-9 was determined as a mixture of peptides derived from β-CN [VPYPQR] (Figs. 20.6A and B) and κ-CN [PPTVERPARNRHD] (Fig. 20.6C). Amino acid sequence of similar κ-CN [PPTVERRPRPRPS] peptide in dromedary camel's was reported by Pauciullo *et al* (2013), and hence genetic variant of κ-CN may exist in Bactrian camel's. Another peptide from $\alpha_{s1}$-casein [RPKYPLR] (Figs. 20.7A and B) was determined in the C3-10. In the last fraction, C3-11, contained the β-CN peptide [QEPVPDPVR] (Figs. 20.8A and B), but its N-terminal glutamine was suspected to be pyroglutamylated by MS/MS that gave 17 Da smaller molecular mass than the theoretical mass. Post-translational modifications such as glycosylation or incomplete separation on RP-HPLC under our experimental conditions may influenced on the analysis of C3-3, -4, -5 and -11, therefore no peptides were identified. In general, among the identified peptides, β-CN fragments were dominant followed by $\alpha_{s1}$-casein whereas fragments from lactophorin and κ-CN were in equal extent (Fig. 18).

The peptides identified in the 22 peaks from fermented mare's milk were summarized in Table 6. Different peptides, [REVERQ] (Figs. 21.1A and B) and [EVSQAKE] (Fig. 21.1C) with same origin from β-casein were found in H1-1 fraction. H1-2 was revealed as a mixture of unknown peptide with mass of 1206 Da and $\alpha_{s2}$-casein peptide [KHNMEHR] (Figs. 21.2A and B), which showed clear spectrum. Peptides derived from $\alpha_{s1}$- and β-CNs determined with amino acid sequence of [AIHAQRK] (Figs. 21.3A and B) and [QREVERQ] (Fig. 21.3C), respectively in the H1-3. In this fraction another peptide whose m/z was 927.4 Da, [QREVERQ] (Fig. 21.3D) but its ammonium ion was disappeared. β-CN peptides with similar sequence, [KFKHEGQQQ] (Figs. 21.4A and B) and [FKHEGQQQR] (Fig. 21.4C) were found in the H1-4. These two peptides were also found in the H1-6 and MS/MS spectrums were totally same (Figs. 21.5A and B). Peptides derived from β-CN [MHQVPQS] (Figs. 21.6A and B) and [RFVQPQP] (Figs. 21.8A and B) detected in the H1-7 and H1-10, respectively. In the H1-8, β-CN fragments were determined such as [MHQVPQ] (Figs. 21.7A and B) and [RDTPVQA] (Fig. 21.7C). A couple of mass signals detected in the H1-13 but only 2 peptides were identified as $\alpha_{s1}$-CN fragment [EYINELNR] (Fig. 21.9C) and κ-CN fragment [QHMPY] (Figs. 21.9A and B). Peak H1-14 was contained as a mixture

of β-CN peptides, [PFPQPVVPYPQ] (Figs. 21.10A and D), [RDTPVQAF] (Fig. 21.10C) and $\alpha_{s1}$-CN peptide [WFHPAQ] (Fig. 21.10B). β-CN fragments, [KLIPTPNGRSLRLPVH] (Fig. 21.11C) and [NLRLPV] (Fig. 21.11B) were detected in the fraction H1-19. A variations of similar peptides from β-CN were observed in the H1-20 ([MLPSQPVLSPPQSKVAPFPQPVPYPQR], Figs. 21.12A, B and [MLPSQPVLSPPQSKVAPFPQPVPYPQRDTPVQ], Fig. 21.12C) and H1-21 ([MLPSQPVLSPPQSKVAPFPQPVPYPQ], Figs. 21.13A and B) and H1-22 ([MLPSQPVLSPPQSKVAPFPQPVPYPQRDTPVQ, Fig. 21.14C). Another short peptide from β-CN, [PPILPF] (Fig. 21.14B) was determined in the H1-22. Post-translational modifications such as glycosylation or incomplete separation on RP-HPLC under our experimental conditions affected the analysis of H1-5, -9, -11, -12, -15, -16, -17 and -18, therefore no peptides were identified. In total, 24 peptides were identified in the ultra-filtrates of fermented mare's milk. Among them, a major peptides were from β-CN followed by $\alpha_{s1}$-CN (Fig. 18).

**III-3. Discussion**

Bioactive peptides can be generated by the starter and non-starter bacteria used for fermentation of the dairy products (Christensen *et al*, 1999). It is well characterized the proteolytic system of lactic acid bacteria (LAB), such as *Lactococcus lactis, Lactobacillus helveticus* and *Lb.delbrueckii* ssp. *Bulgaricus* (Christensen *et al*, 1999). The cell wall-bound proteinase and intracellular peptidases such as endopeptidases, aminopeptidases, tripeptidases and dipeptidases are involved in the proteolytic system of LAB (Christensen *et al*, 1999). Biofuctional peptides with activities of ACE-inhibitory or antihypertensive, immunomodulatory, antioxidative and antimicrobia, released from milk proteins due to microbial proteolysis have been extensively studied and reviewed in the recent past (Gobbetti *et al*, 2002 & 2004; Korhonen & Pihlanto, 2001 & 2004; Matar *et al*, 2003). For the manufacture of traditional fermented milk products, such as Emmental cheese, proteolytic *Lb. helveticus* that can induce the release of bioactive peptides, especially the ACE-inhibitory peptides, is commonly used as a dairy starter. Importantly, proteolysis caused by *Lb. helveticus* strains releases VPP and IPP, which are well-

known ACE-inhibitory peptides (Nakamura *et al*, 1995; Sipola *et al*, 2002). In contrast, ACE-inhibitory activity was not detected when peptides from cheese whey and caseins were analyzed during the manufacture of yoghurt, ropy milk and sour milk using several commercially available dairy starters (Pihlanto-Leppala *et al,* 1998). However, such hydrolysis by digestive and microbial enzymes may release immunomodulatory peptides from milk (Gill *et al,* 2000). For example, a previous study demonstrated that the peptides released from casein when digested with pepsin and trypsin had immunomodulatory effects on lymphocytes from human (Sutas *et al,* 1996). However, such immunomodulatory effects may vary based on the origin of the peptides and the methods used for hydrolysis. For instance, while peptides from total casein and $\alpha_{s1}$-CN after digested by pepsin and trypsin reduced the lymphocyte proliferation, the peptides from β- and κ-CN significantly increased the rate of proliferation. On the other hand, when enzymes from *Lactobacillus* GG var. *casei* were used for hydrolysis before digesting with pepsin and trypsin, hydrolysate fractions from caseins, in particular the $\alpha_{s1}$-CN, were immunosuppressive and found to inhibit the generation of interleukin-4 in lymphocytes (Sutas *et al,* 1996). These findings indicate that the immunological characteristics of milk proteins could be modulated by LAB before or after the oral intake of milk products. The immunosuppressive properties induced by LAB might be beneficial to the consumers with allergies to milk proteins.

Not only the living microbes but also the proteolytic enzymes from LAB may induce the release of antihypertensive and ACE-inhibitory peptides from casein. A previous study found that casein treated with a cell wall-associated proteinase from *Lb. helveticus* CP790 had antihypertensive activity in rats (Yamamoto *et al,* 1994). A subsequent study demonstrated an antihypertensive peptide (KVLPVPQ) derived from β-CN in casein hydrolysed by the same proteinase (Maeno *et al,* 1996). ACE-inhibitory peptides are usually short and often characterized by a proline residue at the C-terminal. Therefore, these ACE-inhibitory short-peptides may be absorbed into blood circulation from the small intestines, as the proline is usually resistant to enzymes in the digestive tract (Yamamoto *et al*, 2003).

The present study is a qualitative analysis on the presence of peptides derived from whey ultra-filtrate isolated from fermented camel's and mare's milks. The PR-HPLC chromatograms of fermented camel's milk reveals that there was a high degree of similarity among the peptide profiles of 3 individual samples (C1, C2 and C3 in Fig. 15), even though those were collected at different locations. Controversially, HPLC profiles among fermented mare's milks seem to be different (H1, H2 and H3 in Fig. 16). In Fig. 17, the representative chromatograms of fermented camel's (C3) and mare's (H1) milk were shown, indicating significant differences between their peptide profiles. The number of peaks in fermented camel's milk were fewer than those of fermented mare's milk.

Probably, all the peptides identified in the both fermented camel's and mare's milks are the product of the indigenous proteases and proteolytic enzymes originated in microorganisms that played important role in the fermentation process. In total, 11 and 24 peptides were identified from fermented camel's and mare's milk, respectively. In the fermented camel's milk, 4 peptides from β-CN, 3 peptides from $\alpha_{s1}$-CN, and 2 peptides from each of κ-CN and lactophorin were identified (Table 5). In case of fermented mare's milk, 19 peptides from β-CN, 3 peptides from $\alpha_{s1}$-CN, and 1 peptides from each of κ-CN and $\alpha_{s2}$-CN were identified (Table 6). It was observed that the ratio of β : $\alpha_{s1}$ : κ : PP3 in fermented camel's milk were 36 : 28 : 18 : 18, whereas ratio of β : $\alpha_{s1}$ : $\alpha_{s2}$ : κ casein in fermented mare's milk were 79 : 13 : 4 : 4. Based this observation, it can be concluded that fermented mare's milk is rich in β-CN fractions followed by $\alpha_{s1}$-CN fraction, while fermented camel's milk contains a mixture of casein fractions equally.

It can be assumed that identified peptides in this study may have several biological activities as below. For example, Muhialdin *et al*, (2018) has recently been reported that peptide mixture of $\alpha_{s1}$-CN f16-20 [RPKYP] and β-CN f1922-199 [QMVPYPQR], f206-219 [VLPFQEPVPDPVRG] and f221-226 [LHPVPQP], isolated from fermented camel's milk shows antibacterial activity against *E. coli, S. aureus, S. faecalis* and *S. dysenteria*. The peptide mixture found in this study, $\alpha_{s1}$-CN f16-22 [RPKYPLR] and β-CN f194-199 [VPYPQR], f210-218 [QEPVPDPVR] f221-226 [HPVPQP] from fermented camel's milk, were partially matched with findings of above study. Therefore, it is highly possible that these newly found peptides may have

antibacterial activity. β-CN f194-199 [VPYPQR] found in this study may have ACE-inhibitory activity (Meisel & Schlimme, 1994). Maeno *et al* (1996) identified a β-CN derived antihypertensive peptide HPVPQP which was found in the peak C3-8 in fermented camel's milk. β- and α-CN fragments are known to encrypt opioid agonists (β-casomorphins), ACE inhibitory activity (β-casokinins), antibacterial, antioxidant, mineral binding and immunostimulating activities (Anne *et al*, 1998; Christian *et al*, 2007; Martha *et al*, 2009).

The most specific feature of peptides found in the fermented camel's milk was presence of κ-CN- and lactophorin-derived peptides in significant amounts (Fig. 18 and Table 5). The κ-CN-derived peptide [PPTVERPARNRHD] in C3-9 was similar to [PPTVERRPRPRPS] at the C-terminal region of Bactrian camel's κ-CN. C-terminal region of κ-CN peptide [RPRPRPS] was detected in C3-6 and C3-7. And hence, peptide sequence in C3-9 might be [PPTVERRPRPRPS]. The other peptides, especially identified in the fermented mare's milk in this study, were not found in any of previous studies.

The mass of 9 peptides in fermented camel's milk (Table 5) and 36 peptides (Table 6) in fermented mare's milk were measured, but not identified yet, even though mass spectrums were clear (data not shown). This is probably because of post translational modifications (PTM), such as glycosylation, oxidation and phosphorylation occurred in camel's and cow's colostrum. Glycosylation occurs naturally in milk proteins, with examples of both *N*- and *O*- linked glycoproteins (O'Donnell *et al*, 2004). *N*-Linked glycosylation of asparagine residues has been associated with numerous milk proteins (O'Donnell *et al*, 2004). It is particularly prevalent in proteins of the milk fat globule membrane (MFGM) (O'Donnell *et al*, 2004). Glycosylation can occur at serine and threonine residues through *O*-linked glycosylation (O'Donnell *et al*, 2004). However, the glycosylation was not detected in identified peptides by mass analyses in this study. But hydroxylation of a glutamine residue and phosphorylation at a serine residue were detected by MS in the peptides derived from β-CN [QEPVDPVR] and β-CN [LIPTPNGRSLRLPVH], respectively.

Bioactive peptides can be incorporated in the form of ingredients in novel functional foods, dietary supplements and even pharmaceuticals with purpose of delivering specific health benefits.

Therefore, further investigation is needed in order to demonstrate biological activity of peptides identified in this study, e.g. by demonstrating biological assays using chemically synthesized peptides. In addition, reproducibility of peptide extraction from the traditional fermented milks as performed in this study should be confirmed.

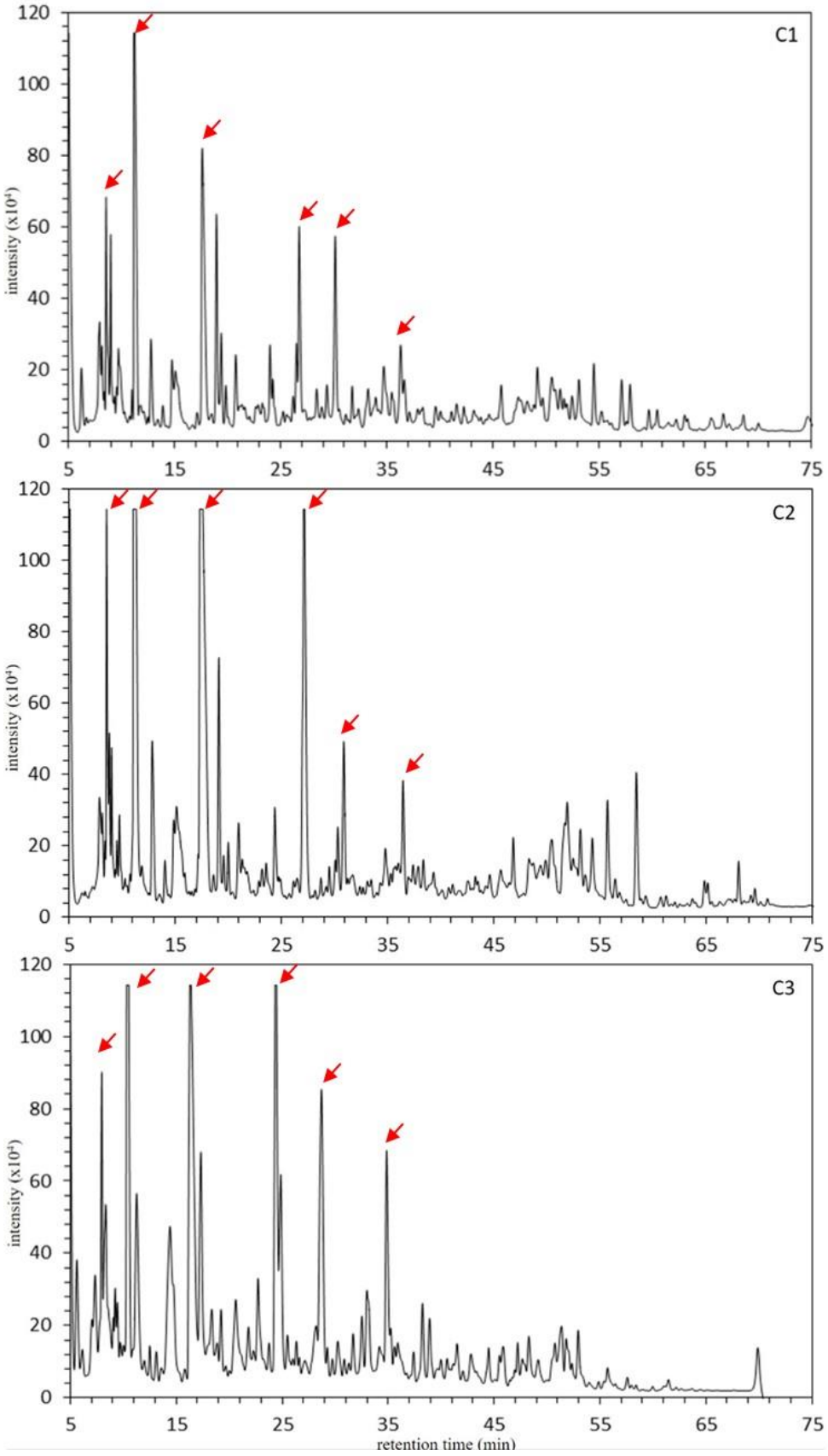


Figure 15. RP-HPLC profile of ultra-filtrates (NMWL 3,000) of fermented camel's milk (sample number; C1, C2 and C3).

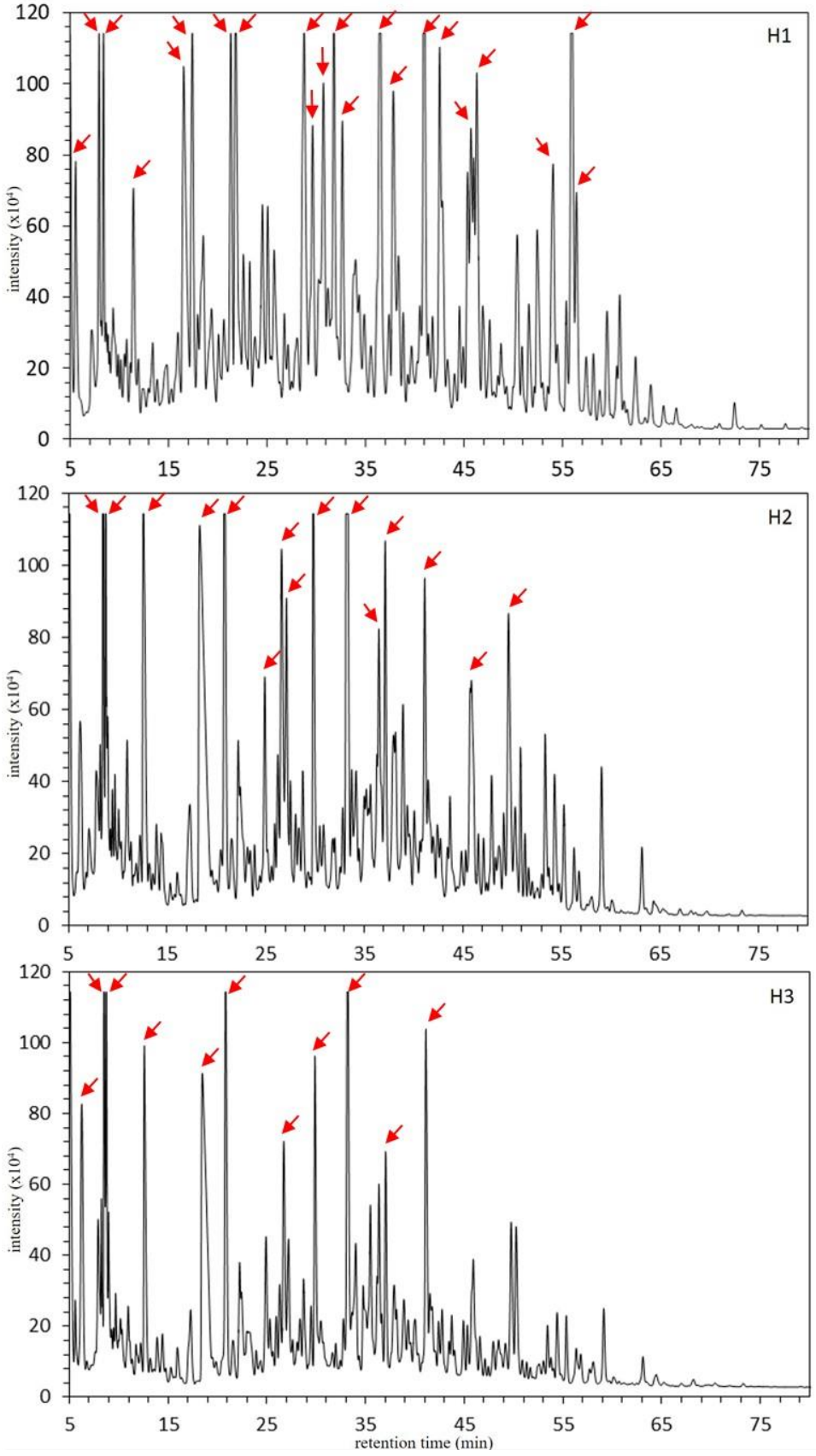


Figure 16. RP-HPLC profile of ultra-filtrates (NMWL 3,000) of fermented mare's milk (sample number; H1, H2 and H3).

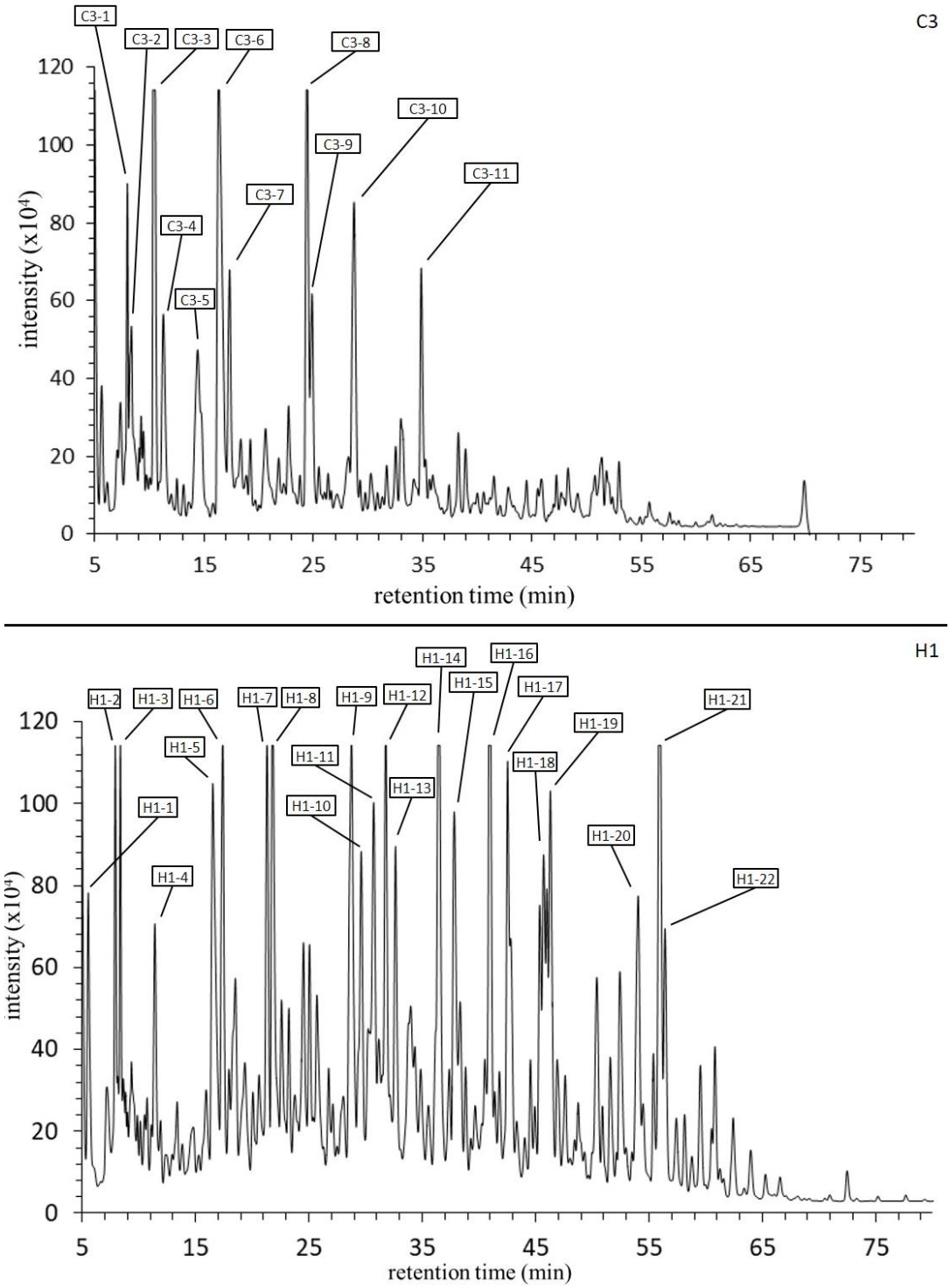


Figure 17. Peak fractionation by RP-HPLC of fermented milk whey ultra-filtrates (NMWL 3,000) from camel's (C3) and mare's (H1) milk. Fractionated peaks were numbered.

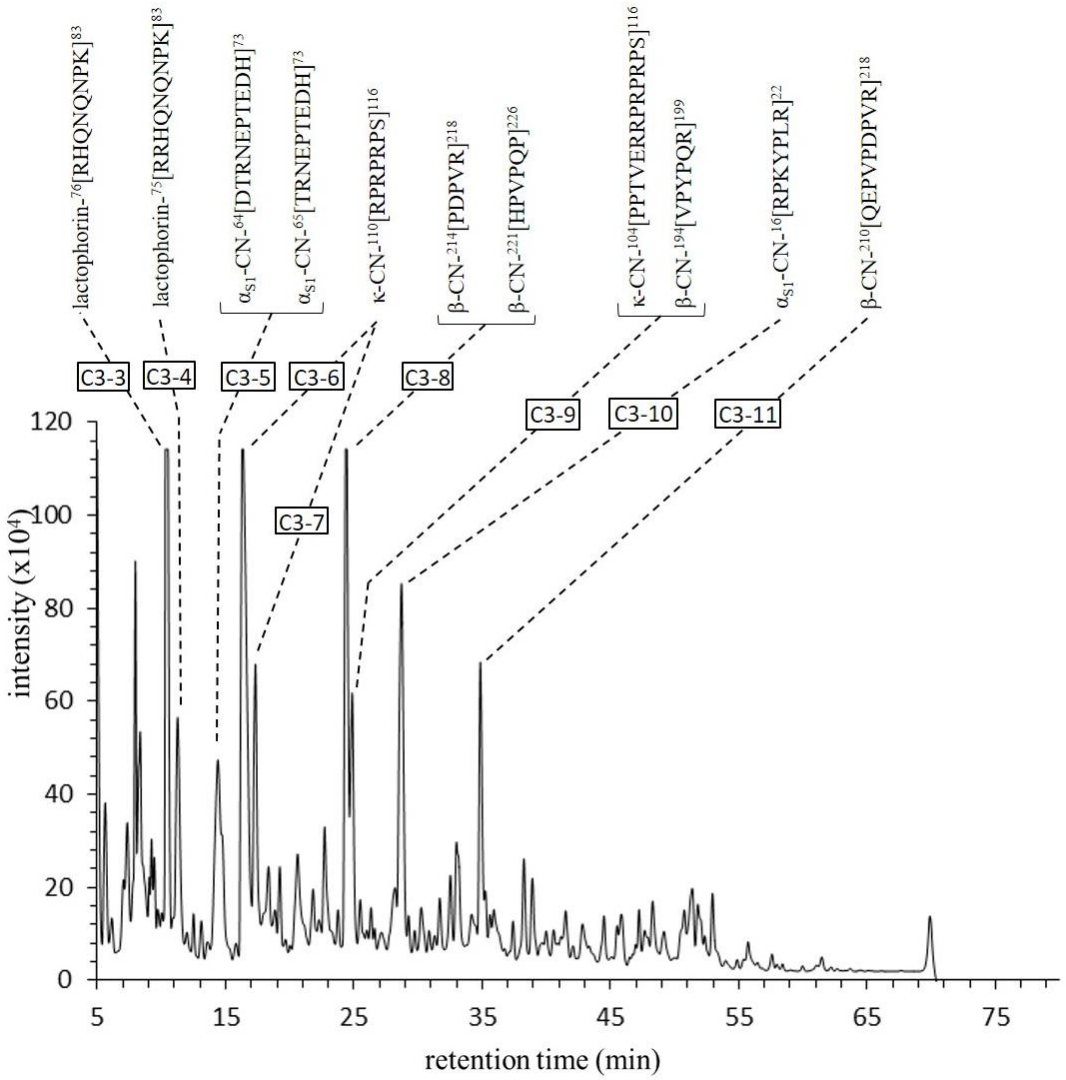


Figure 18. Identified peptides in the ultra-filtrates (NMWL 3,000) of fermented camel's milk whey.

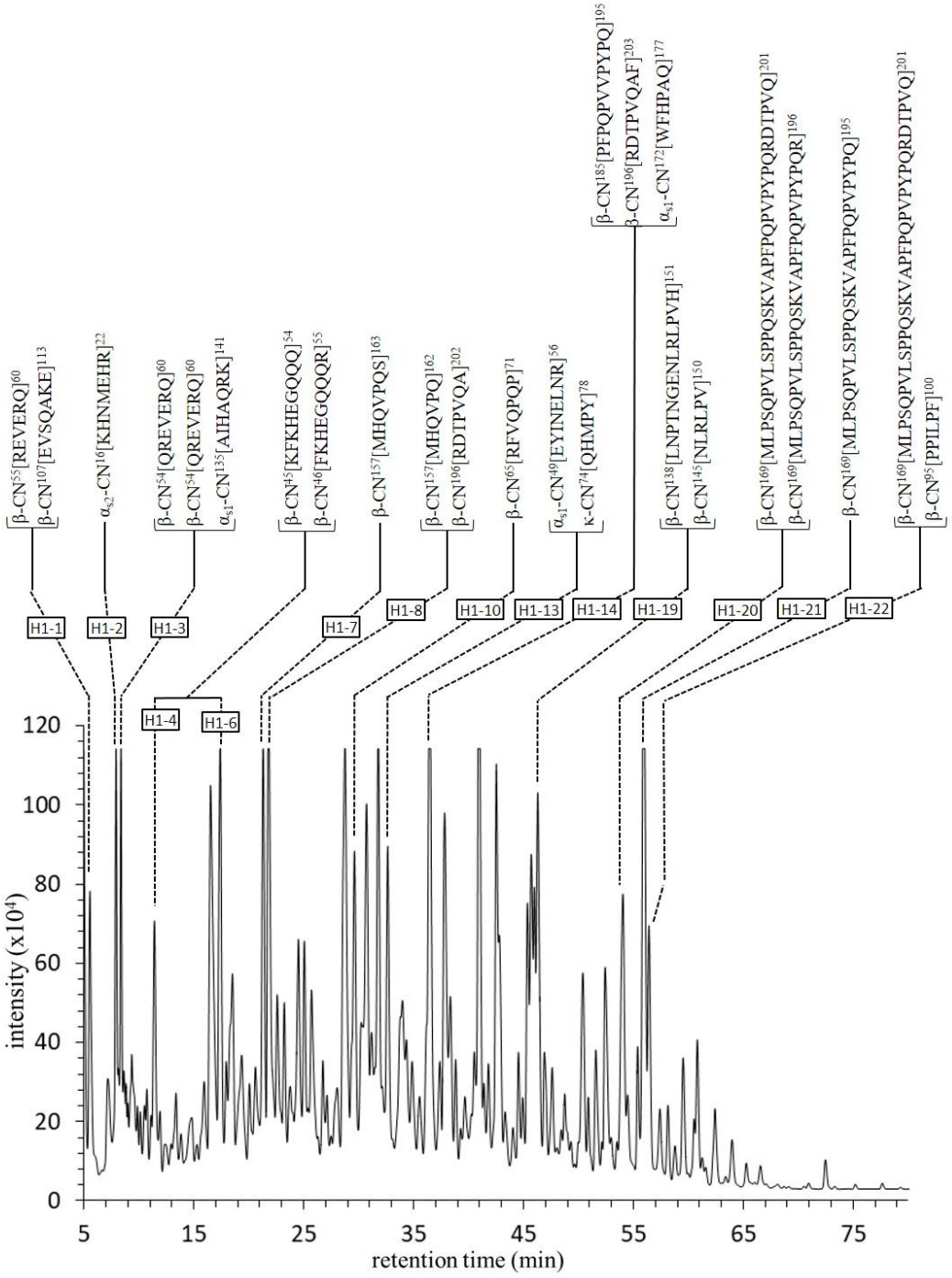


Figure 19. Identified peptides in the ultra-filtrates (NMWL 3,000) of fermented mare's milk whey.

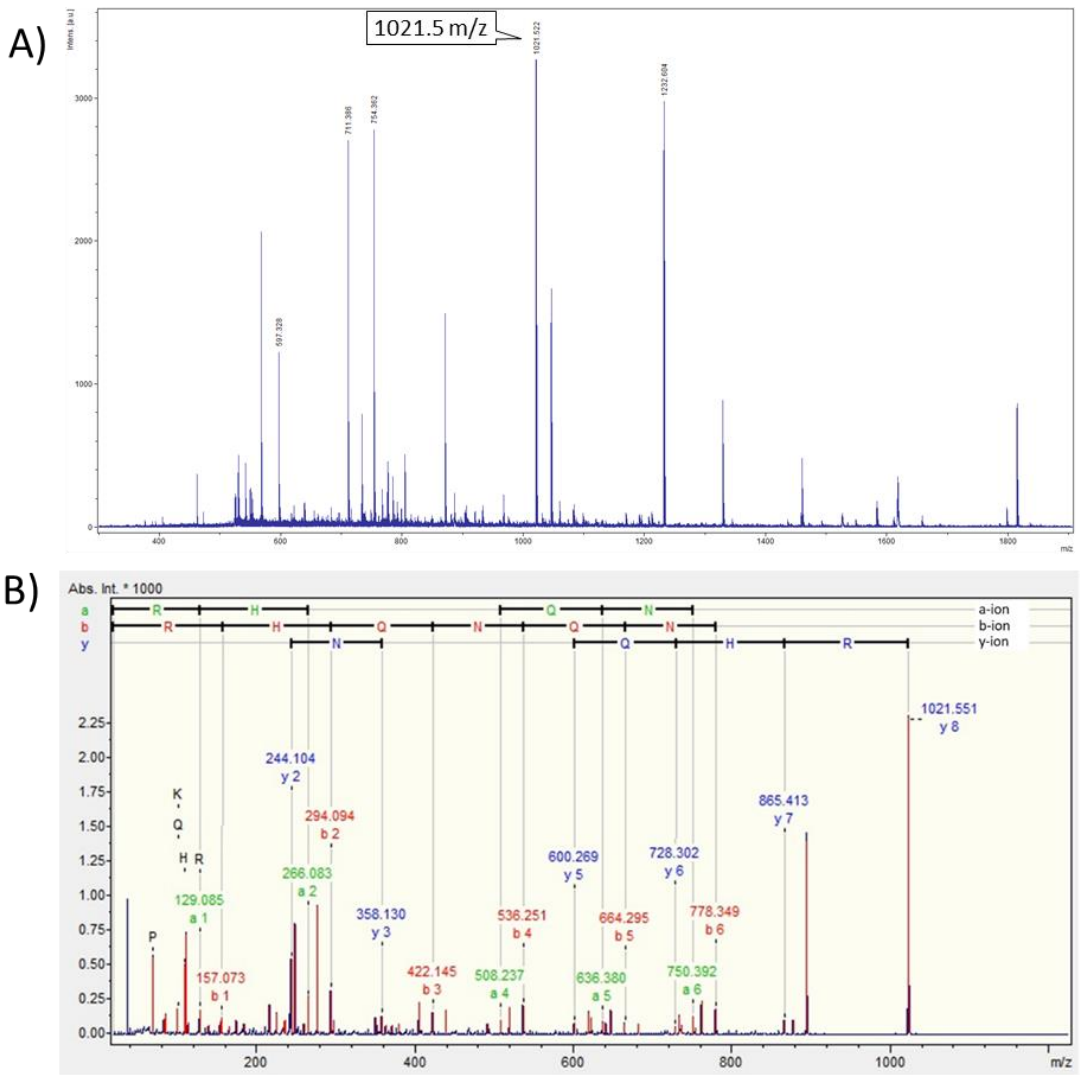


Fig. 20.1. MS spectrum of the parent peak, m/z 1021.522 (A) detected in C3-3. The parent peak was identified as a PP3 (f76-83) [RHQNQNPK] (B) of the fermented camel milk by MS/MS in Table 5.

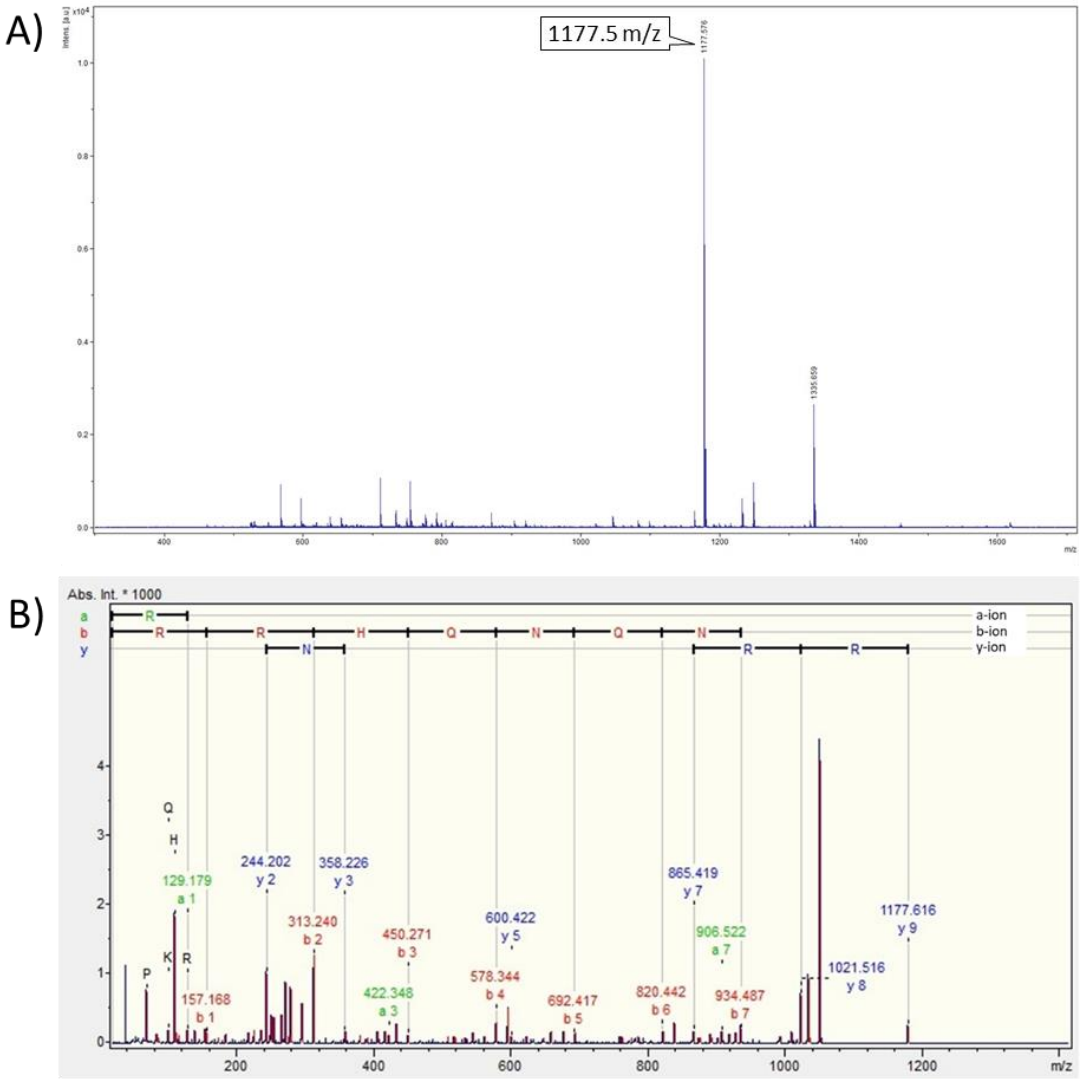


Fig. 20.2. MS spectrum of the parent peak, m/z 1177.576 detected in C3-4. The parent peak was identified as a PP3 (f75-83) [RRHQNQNPK] (B) of the fermented camel milk by MS/MS in Table 5.

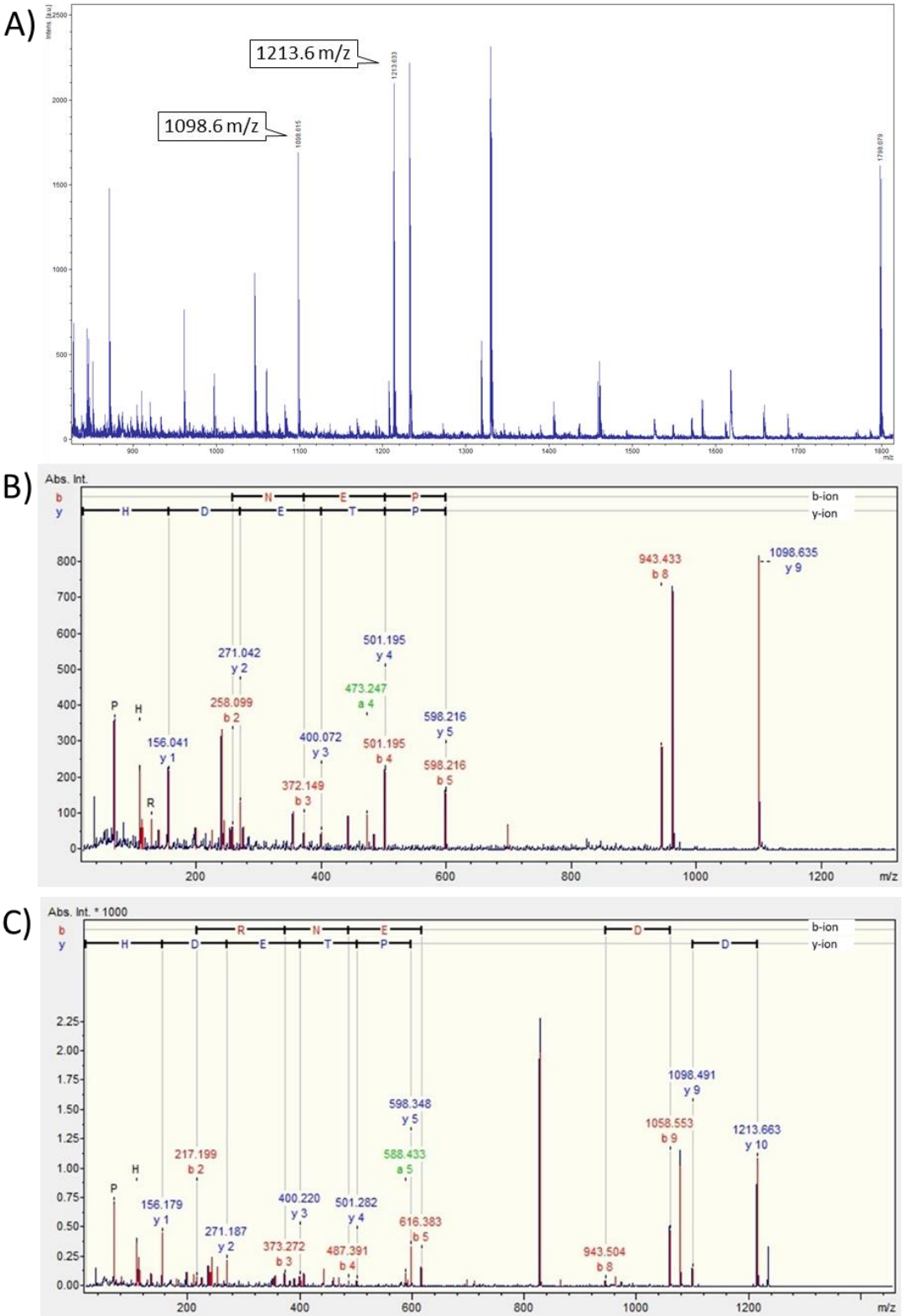


Fig. 20.3. MS spectrums of the parent peak, m/z 1098.615 and 1213.633 detected in C3-5 (A). MS/MS spectrum of the peak m/z 1098.6 was identified as $\alpha_{s1}$-CN (f65-73) [TRNEPTEDH] (B) and peak C3-5, m/z 1213.6 was $\alpha_{s1}$-CN (f64-73) [DTRNEPTEDH] (C), respectively.

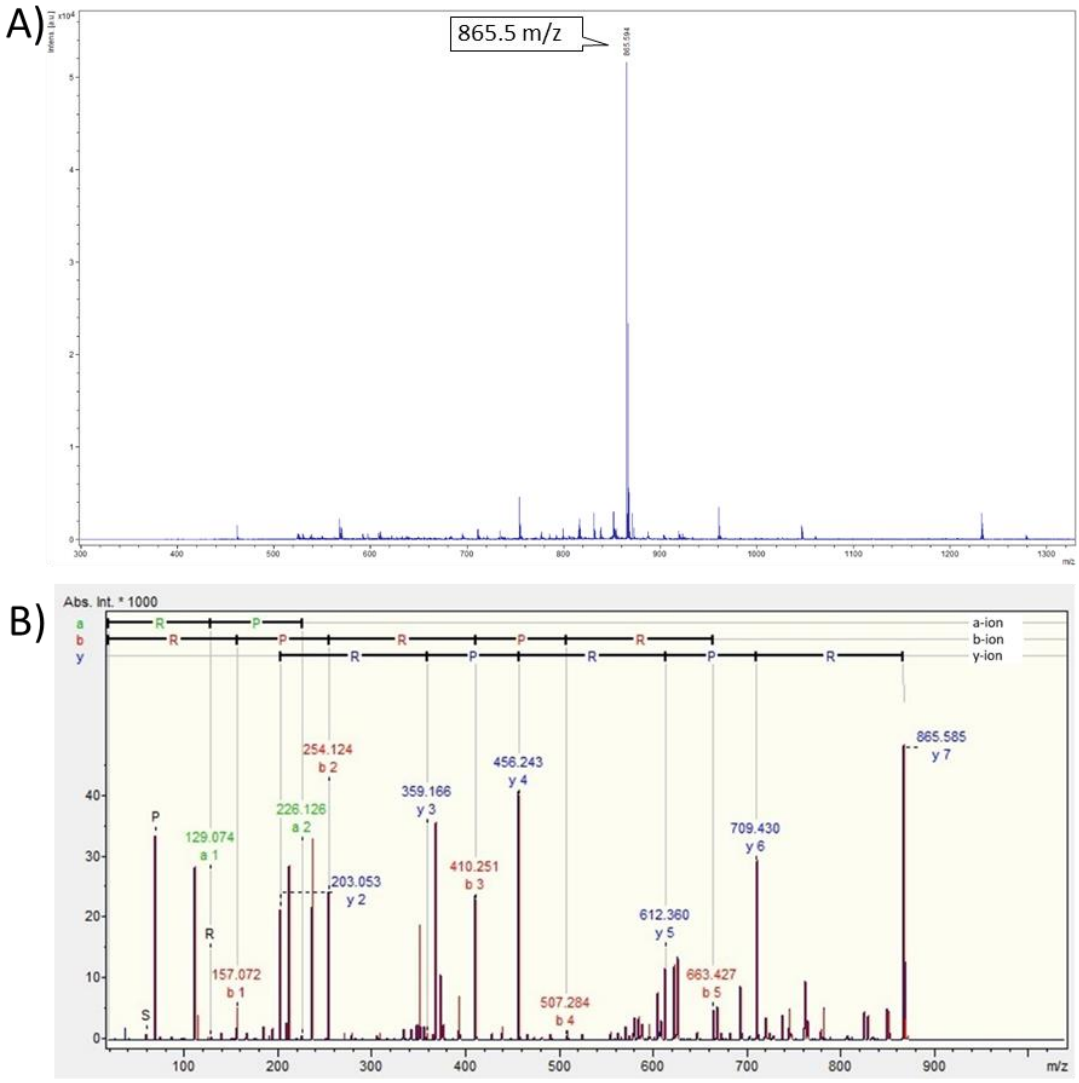


Fig. 20.4. MS spectrum of the parent peak, m/z 865.5 detected in C3-6 and C3-7 (A). The parent peak was identified as κ-CN (f110-116) [RPRPRPS] (B) by MS/MS.

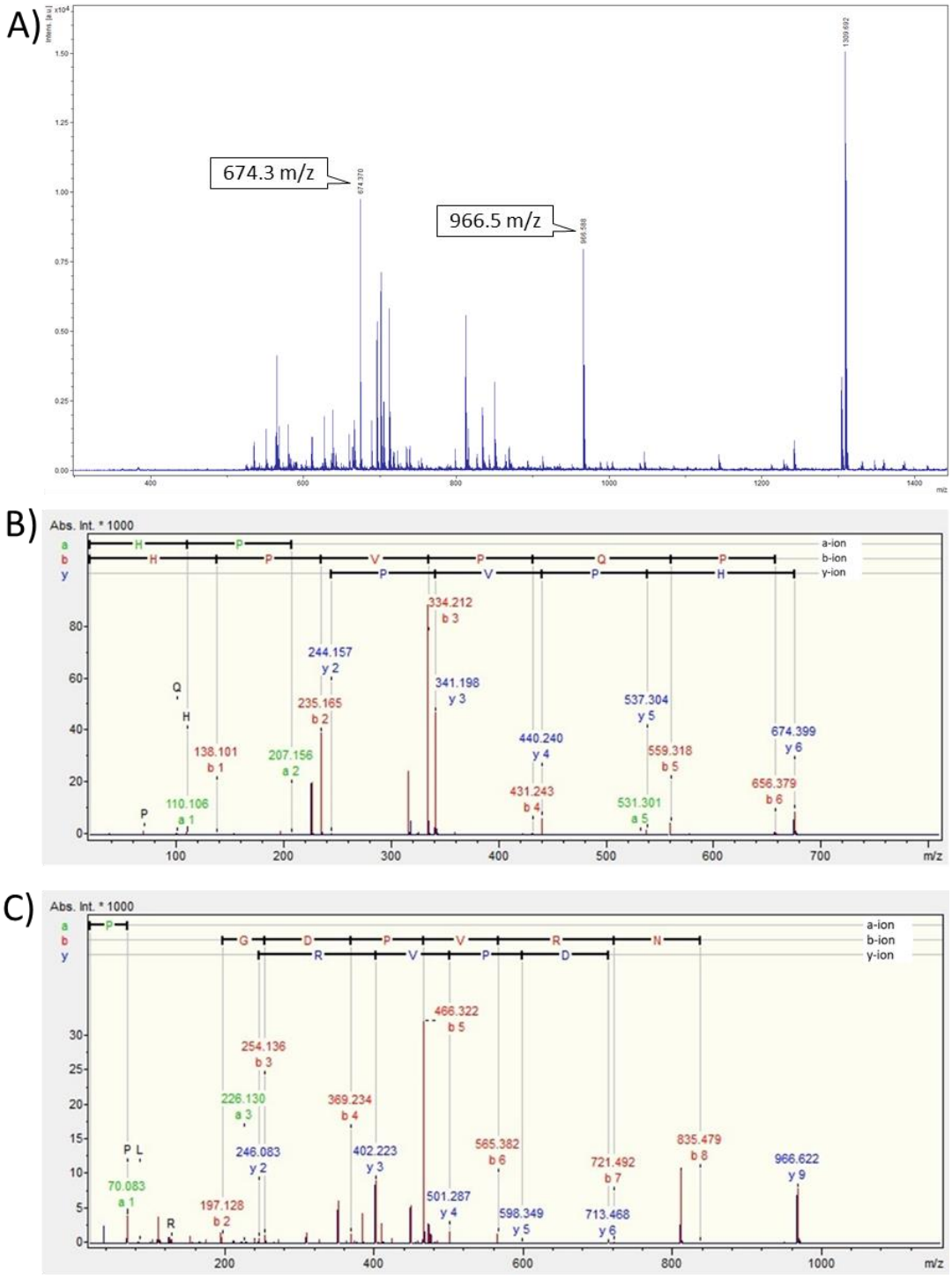


Fig. 20.5. MS spectrums of the parent peak, m/z 674.3 and 966.5 detected in C3-8 (A). The parent peak m/z 674.3 as β-CN (f221-226) [HPVPQP] (B) and 966.5 β-CN (f214-218) [PDPVR] (C) by MS/MS in Table 5.

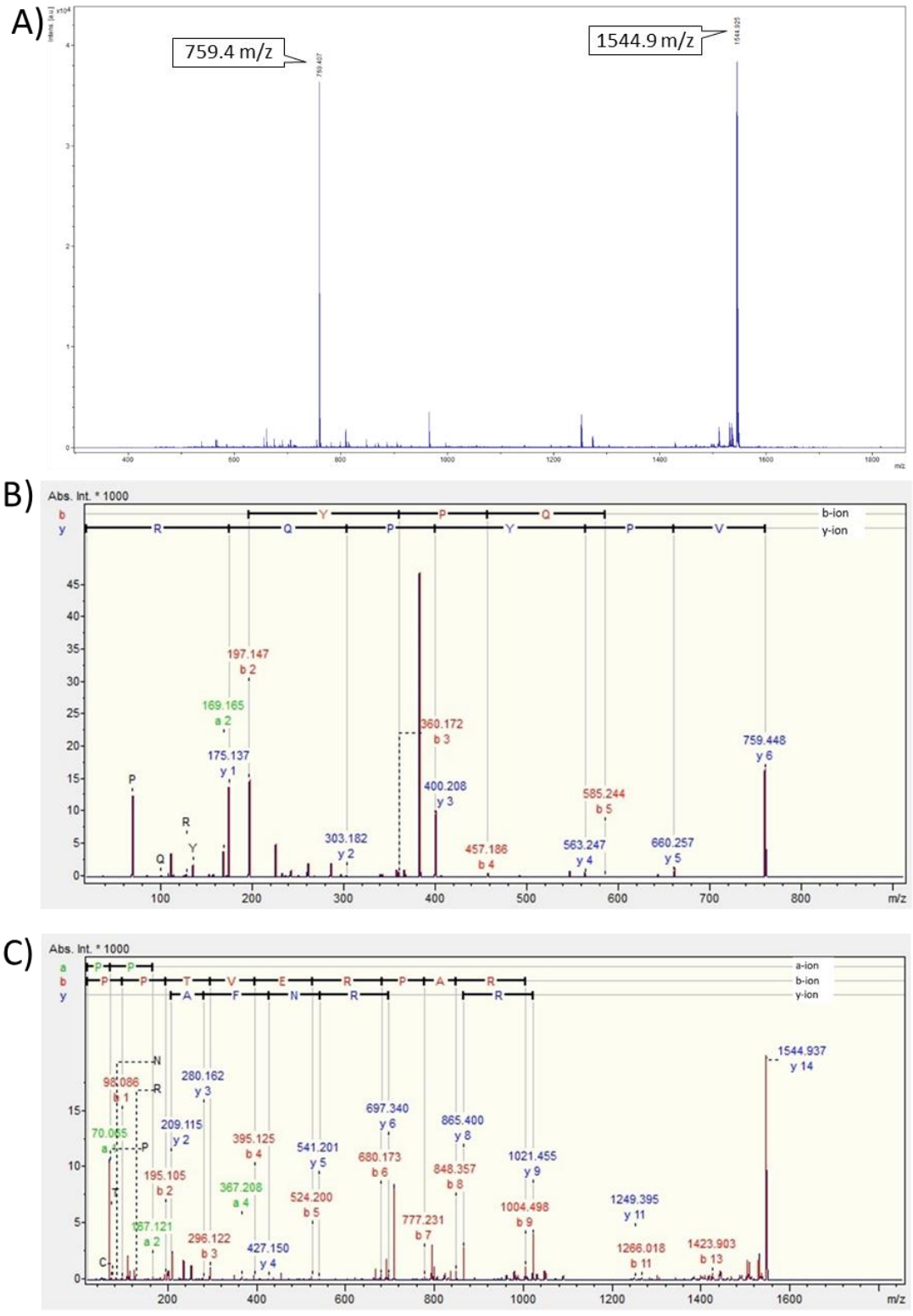


Fig. 20.6. MS spectrum of the parent peaks, m/z 759.4 and 1544.9 detected in C3-9 (A). MS/MS spectrum of the peak m/z 759.4 was β-CN (f194-199) [VPYPQR] (B) and m/z 1544.9 was κ-CN (f104-116) [PPTVERRPRPRPS] (C), respectively (Table 5).

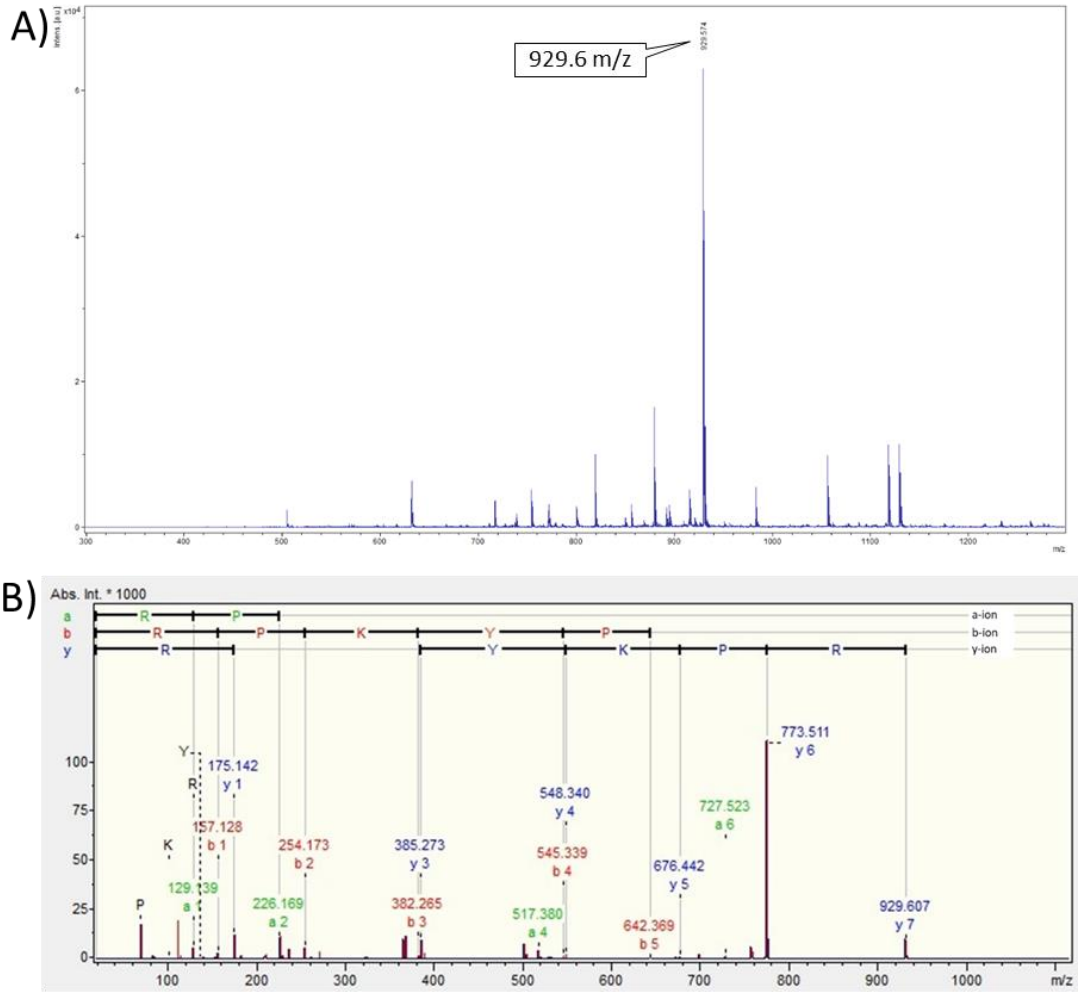


Fig. 20.7. MS spectrum of the parent peak, m/z 929.5 detected in C3-10 (A). The parent peak was identified as $\alpha_{s1}$-CN (f16-22) [RPKYPLR] by MS/MS in Table 5.

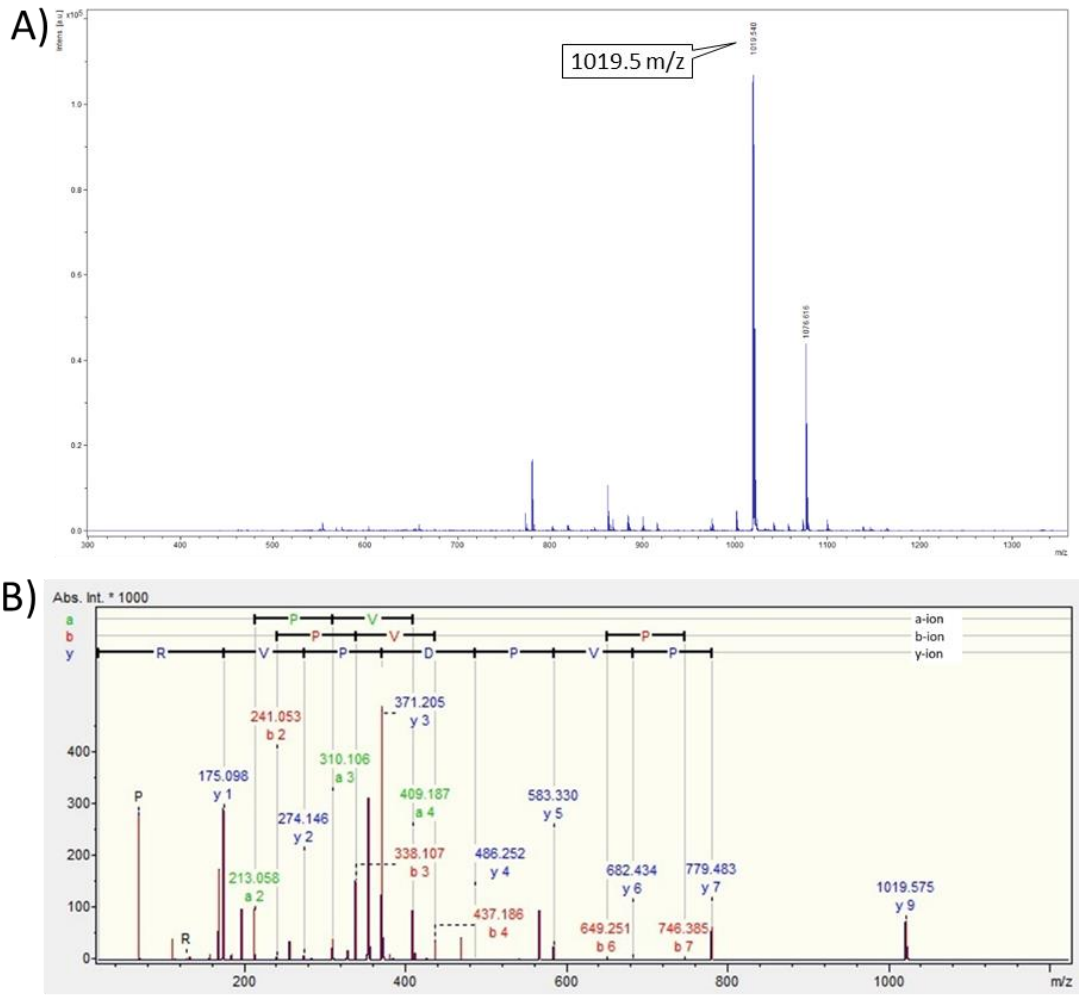


Fig. 20.8. MS spectrum of the parent peak, peak m/z 1019.5 Da detected in C3-11 (A) and peptide sequence was β-CN (f210-218) [QEPVPDPVR] (B) of the fermented camel milk (Table 5).

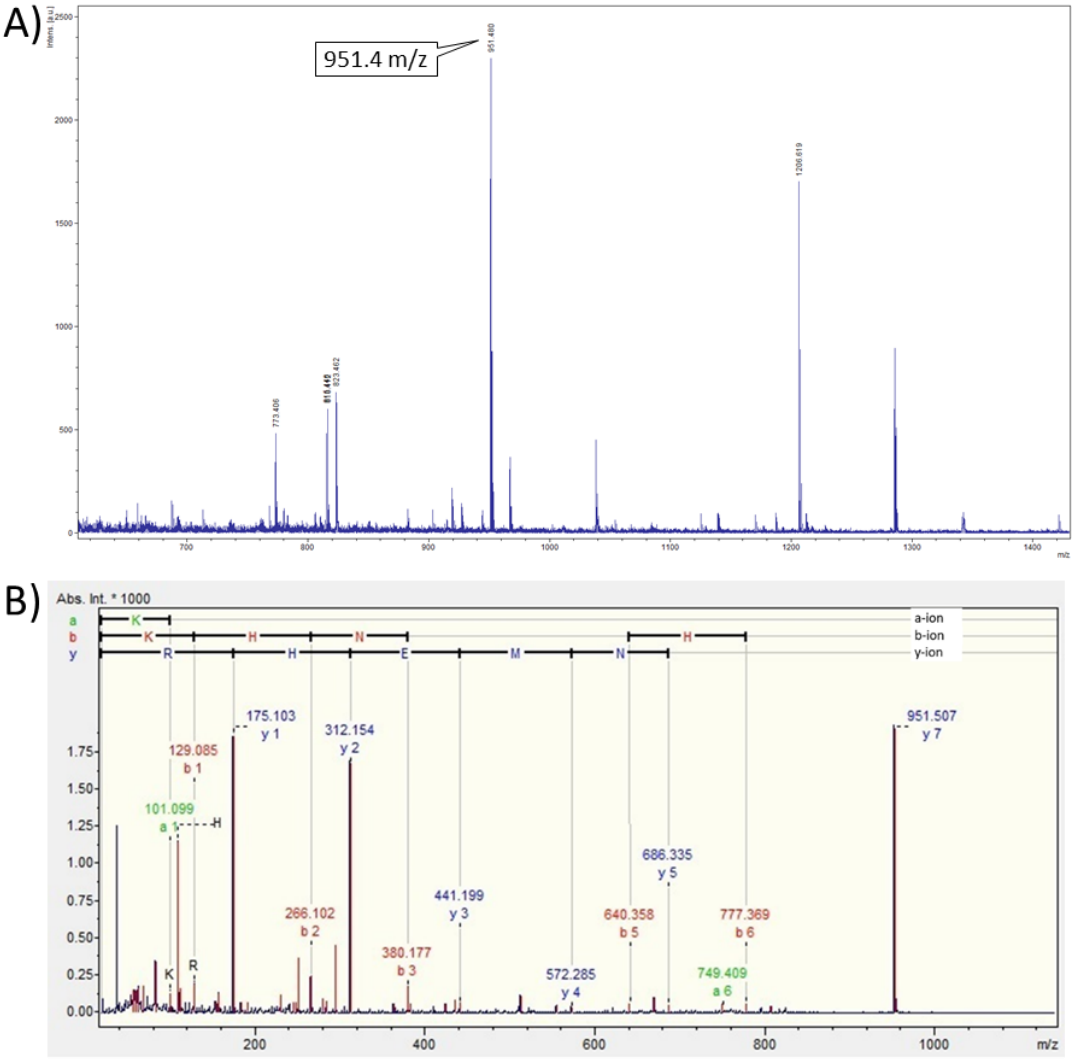


Fig. 21.2. MS spectrum of the parent peak m/z 951.4 detected in H1-2 (A). MS/MS spectrum of the peak H1-2, m/z 951.4 was $\alpha_{s2}$-CN f16-22 [KHNMEHR] (B) in Table 6.

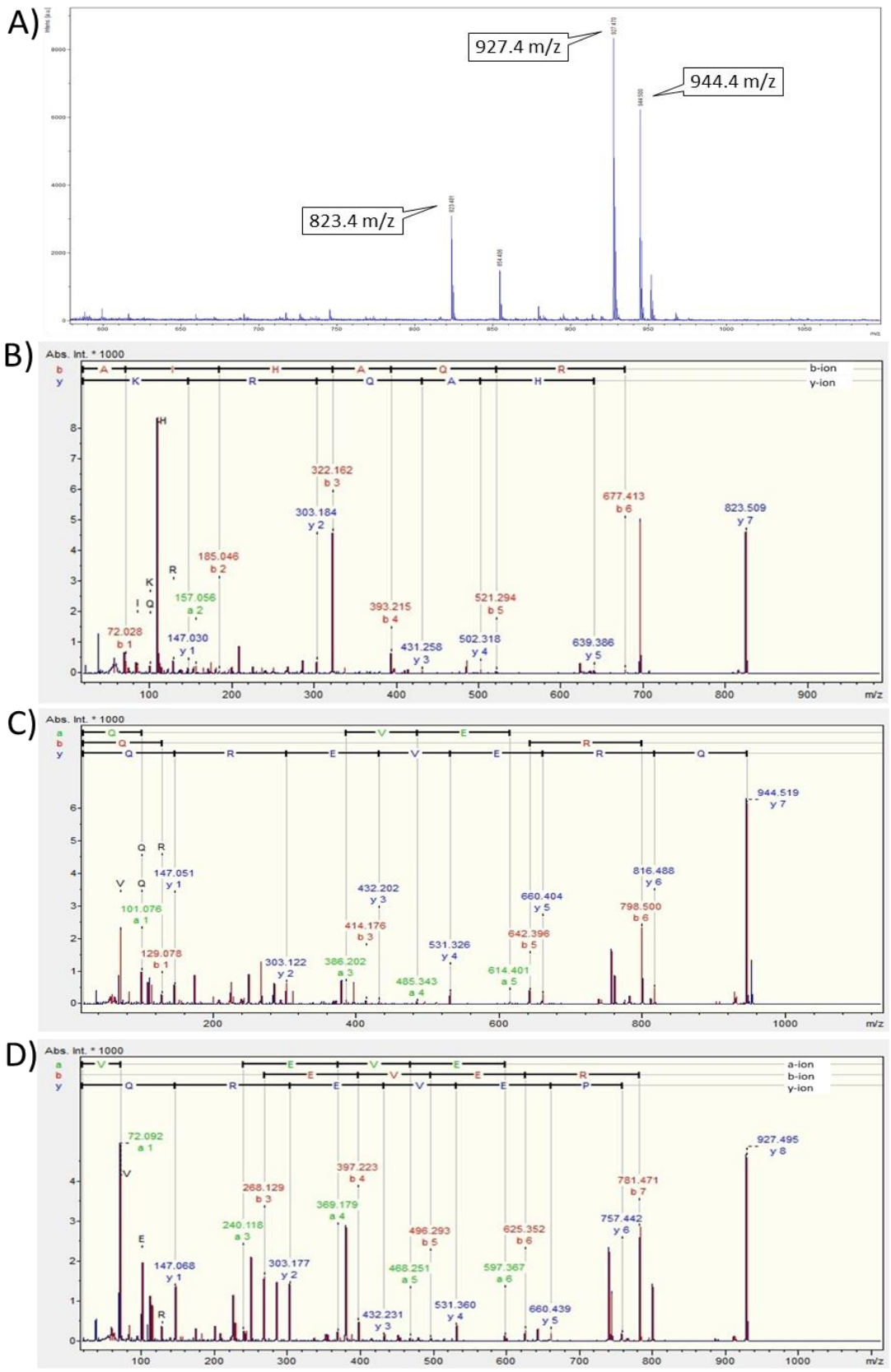


Fig. 21.3. MS spectrum of the parent peaks, m/z 823.4, 944.4 and 927.4 detected in H1-3 (A). MS/MS spectrums of the peak H1-3 were m/z 823.4 $\alpha_{s1}$-CN f135-141 [AIHAQRK] (B), , m/z 944.4 Da for β-CN f62-68 [QREVERQ] (C) and m/z 927.4 Da for β-CN f62-68 [QREVERQ], in Table 6.

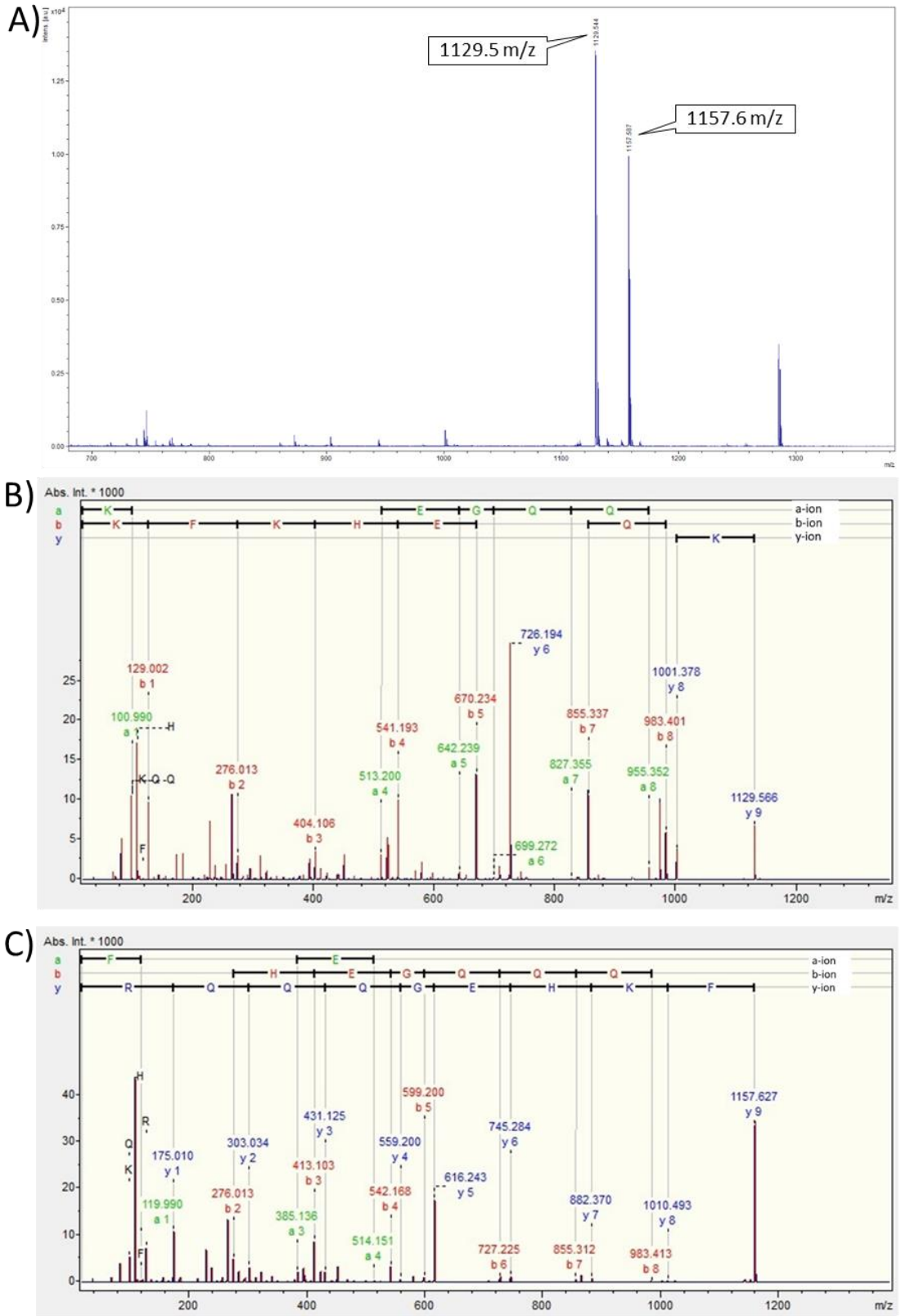


Fig. 21.4. MS spectrum of the parent peaks, m/z 1129.5 and 1157.5 detected in H1-4 (A). MS/MS spectrum of the peak H1-4, m/z 1129.5 Da for β-CN f45-54 [KFKHEGQQQ] (B) and m/z 1157.5 Da for β-CN f46-55 [FKHEGQQQR] (C), in Table 6.

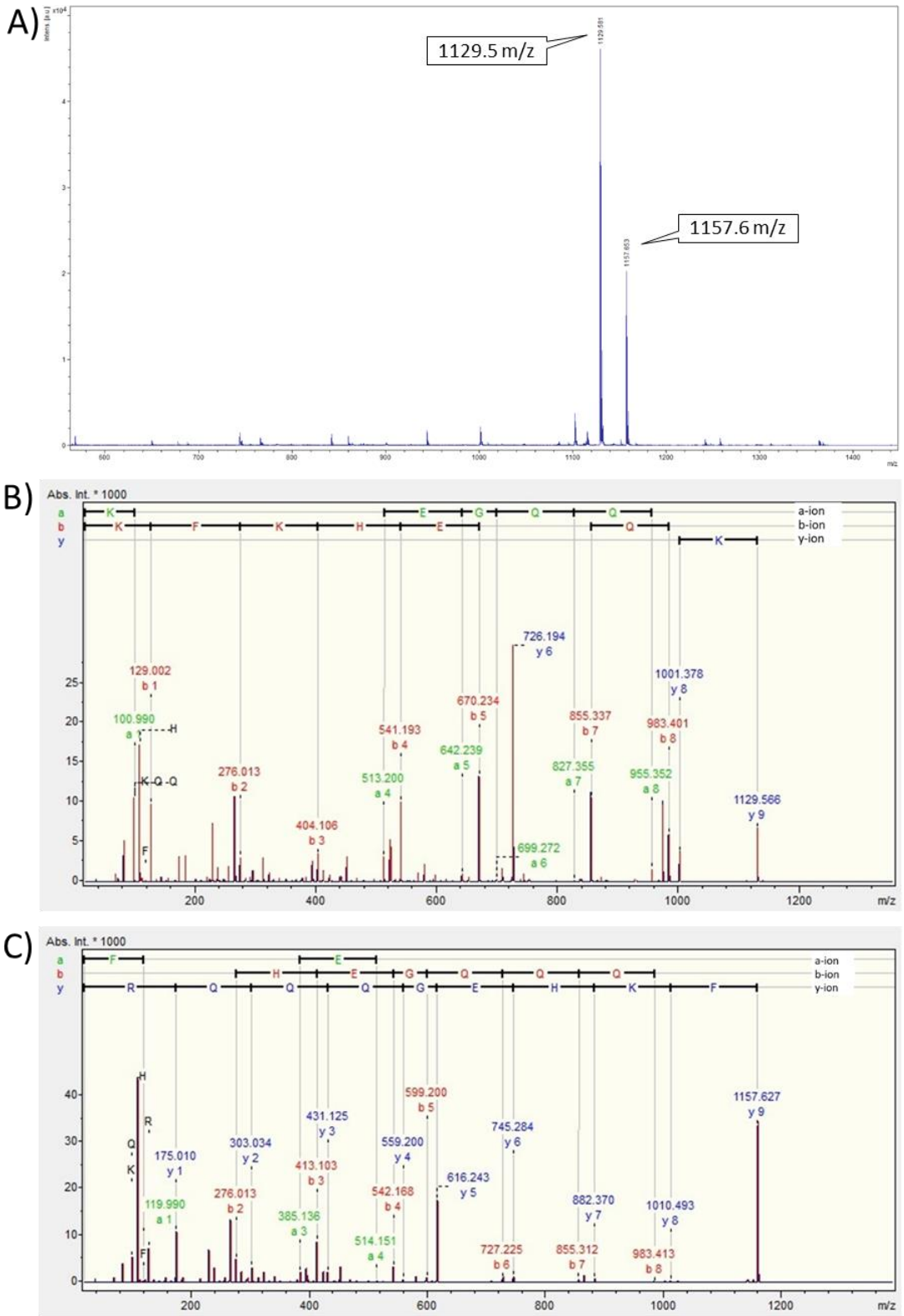


Fig. 21.5. MS spectrum of the parent peaks, m/z 1129.5 and 1157.5 detected in H1-6 (A). MS/MS spectrum of the peak H1-6, m/z 1129.5 Da for β-CN f45-54 [KFKHEGQQQ] (B) and m/z 1157.5 Da for β-CN f46-55 [FKHEGQQQR] (C), in Table 6.

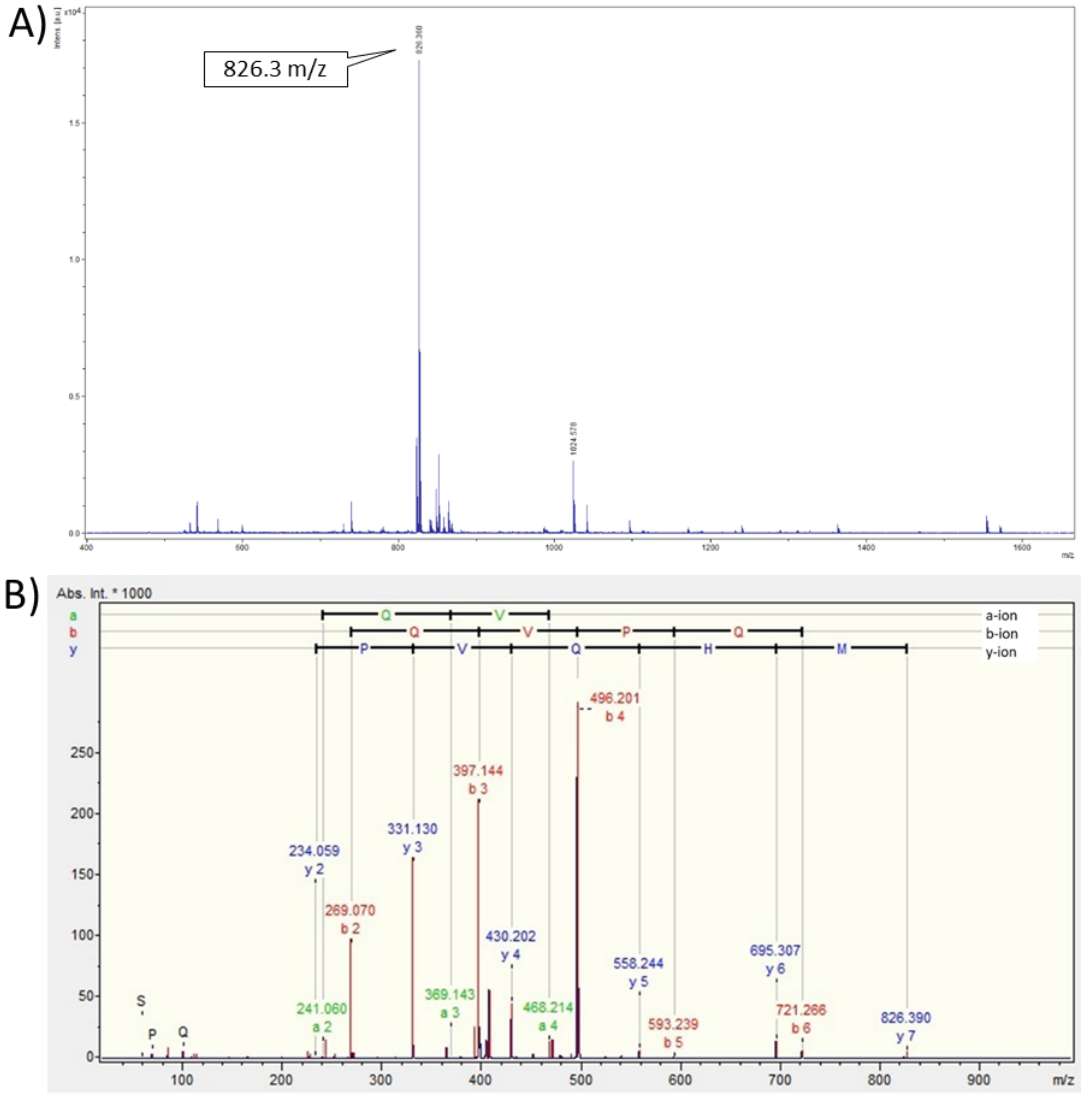


Fig. 21.6. MS spectrum of the parent peak, m/z 826.3 detected in H1-7 (A). MS/MS spectrum of the peak H1-7 m/z 826.3 was β-CN f157-163 [MHQVPQS] (B) in Table 6.

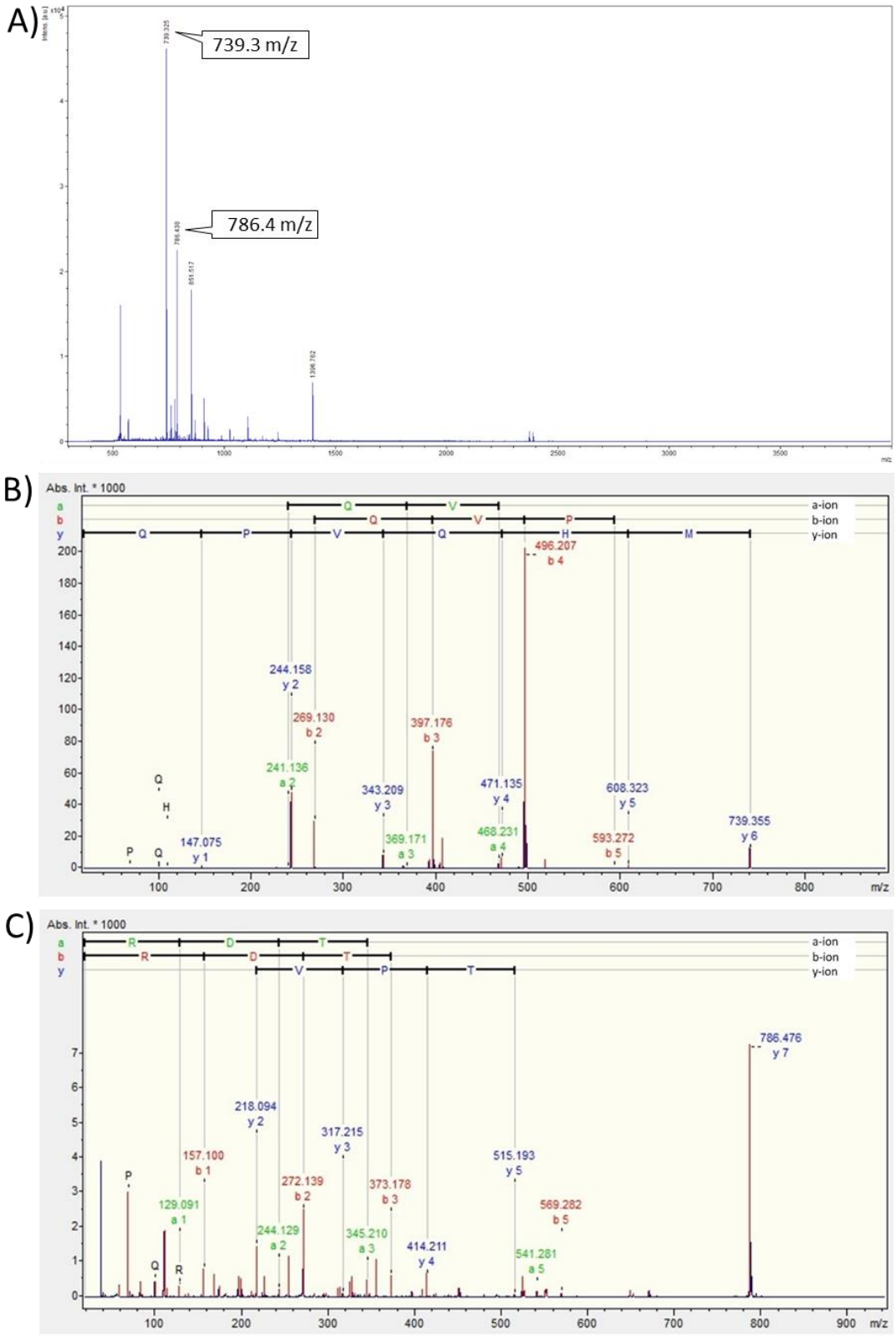


Fig. 21.7. MS spectrum of the parent peak, m/z 739.3 detected in H1-8 (A). MS/MS spectrum of the peak H1-8 was m/z 739.3, β-CN f157-162 [MHQVPQ] (B) and m/z 786.4, β-CN f196-202 [RDTPVQA] (C), in Table 6.

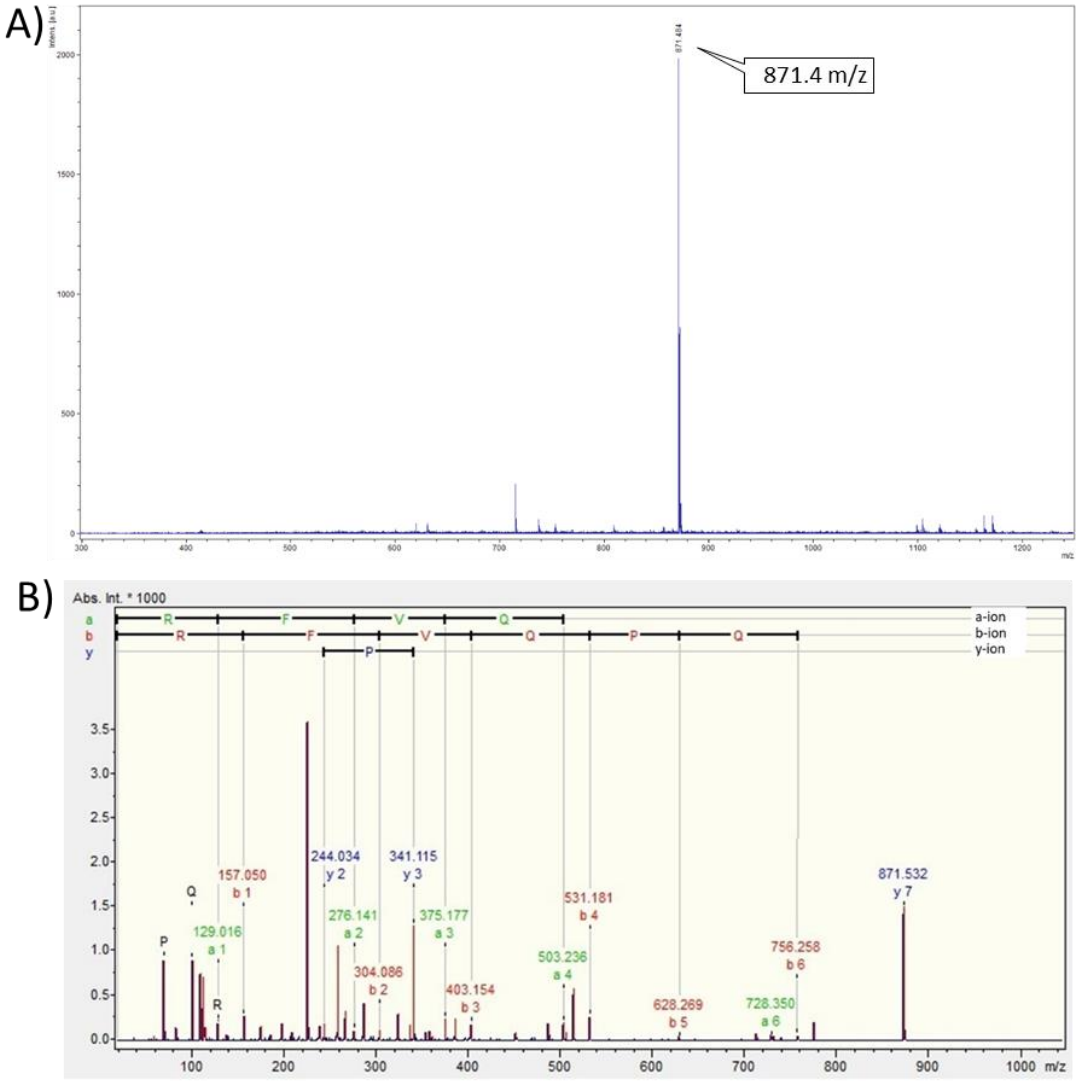


Fig. 21.8. MS spectrum of the parent peak, m/z 871.4 detected in H1-10 (A). MS/MS spectrum of the peak H1-10 m/z 871.4 Da for β-CN f65-71 [RFVQPQP] (B) in Table 6.

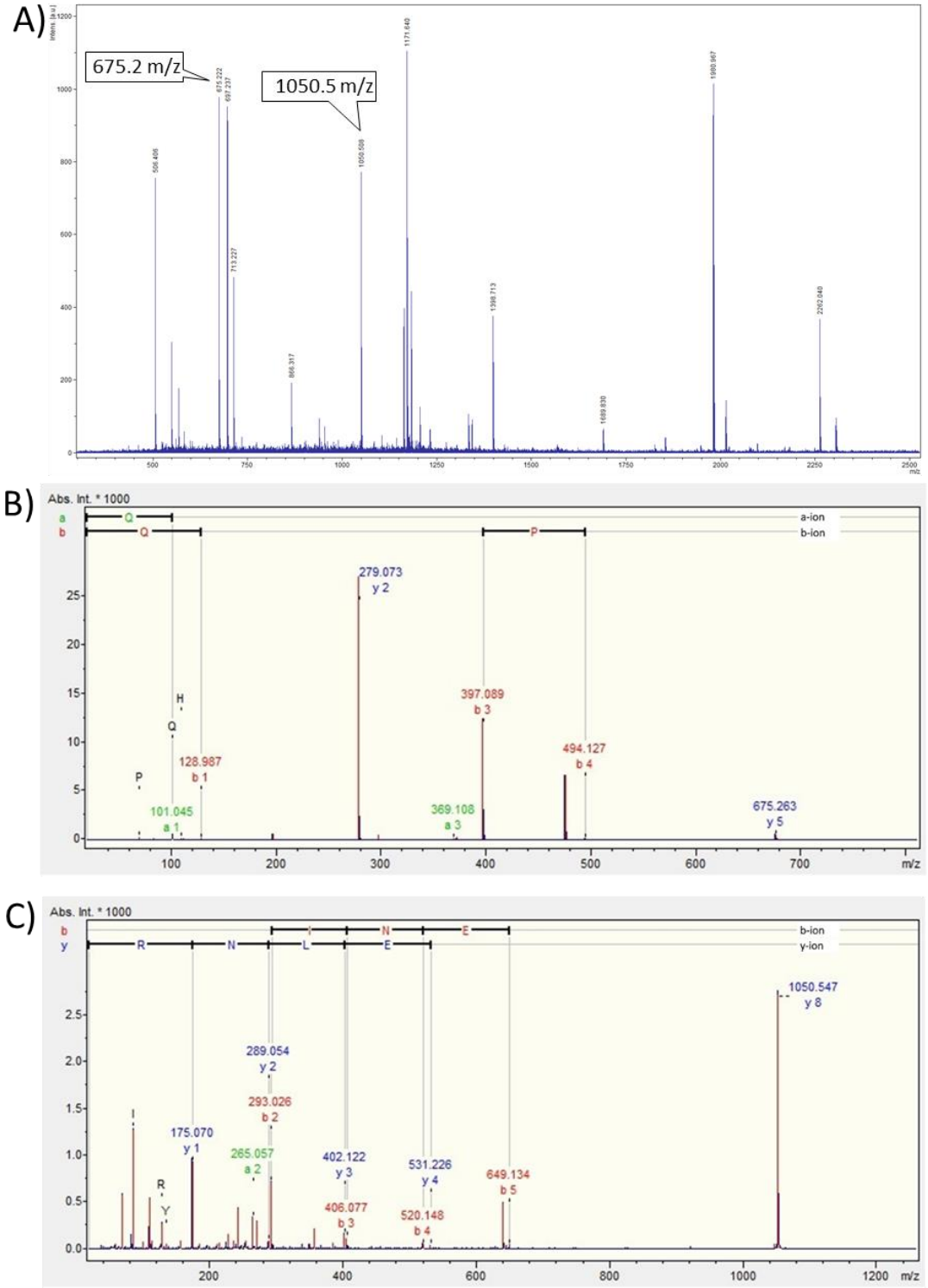


Fig. 21.9. MS spectrum of the parent peaks, m/z 675.2 and 1050.5 detected in H1-13 (A). MS/MS spectrum of the peak H1-13 was m/z 675.2, κ-CN f74-78 [QHMPY] (B) and m/z 1050.5, $\alpha_{s1}$-CN f49-56 [EYINELNR] (C) in Table 6.

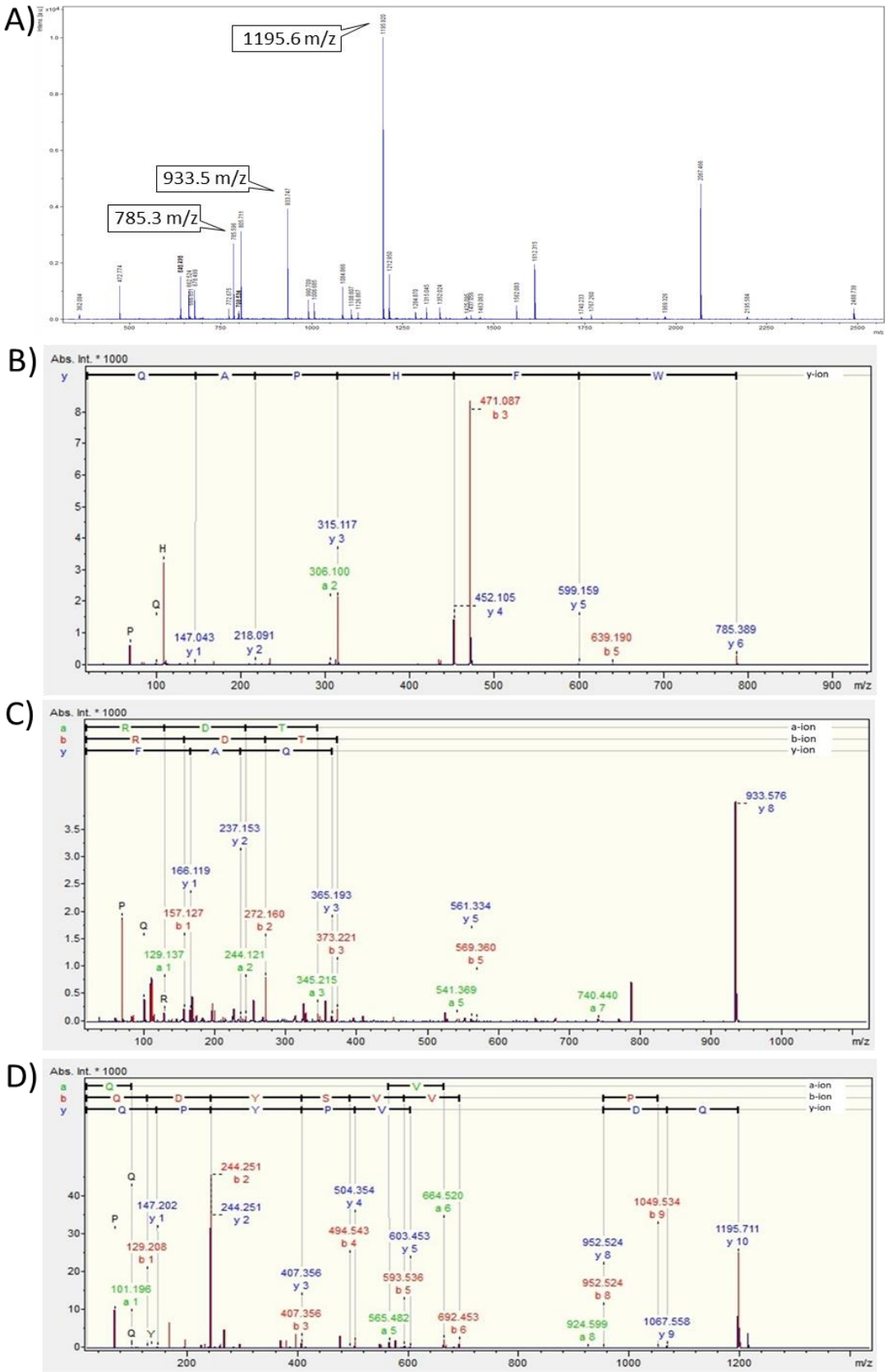


Fig. 21.10. MS spectrum of the parent peak, m/z 785.3, 933.5 and 1195.6 detected in H1-14 (A). MS/MS spectrums of the peak H1-14 were m/z 785.3,$\alpha_{s1}$-CN f172-177 [WFHPAQ] (B); m/z 933.5, β-CN f196-203 [RDTPVQAF] (C) and m/z 1195.6, β-CN f185-195 [PFPQPVVPYPQ] (D) in Table 6.

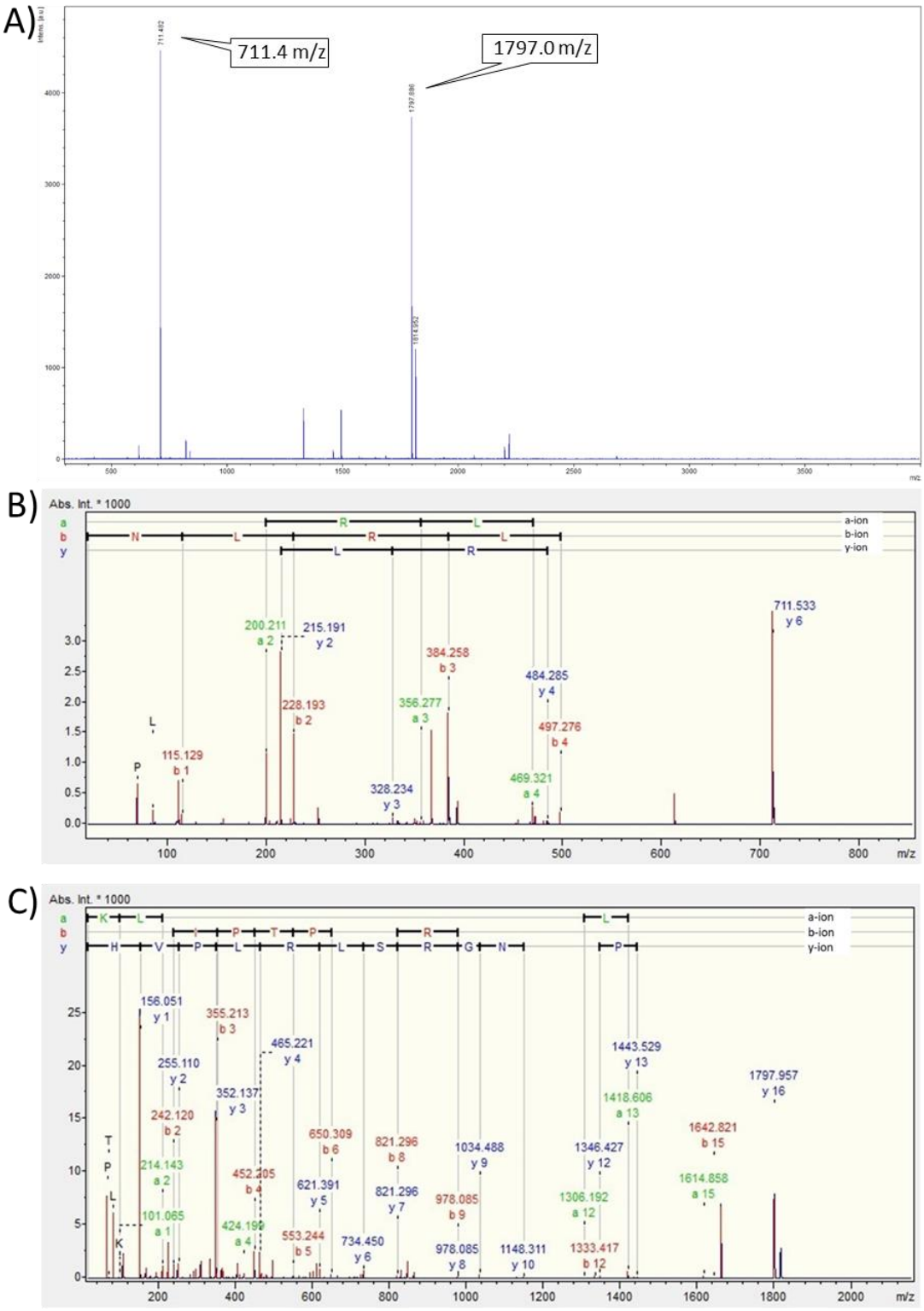


Fig. 21.11. MS spectrum of the parent peaks, m/z 711.4 and 1797.0 detected in H1-19 (A). MS/MS spectrums of the peak H1-19 were: m/z 711.4, β-CN f145-150 [NLRLPV] (B) and m/z 1797.0, β-CN f138-151 [KLIPTPNGRSLRLPVH] (C), in Table 6.

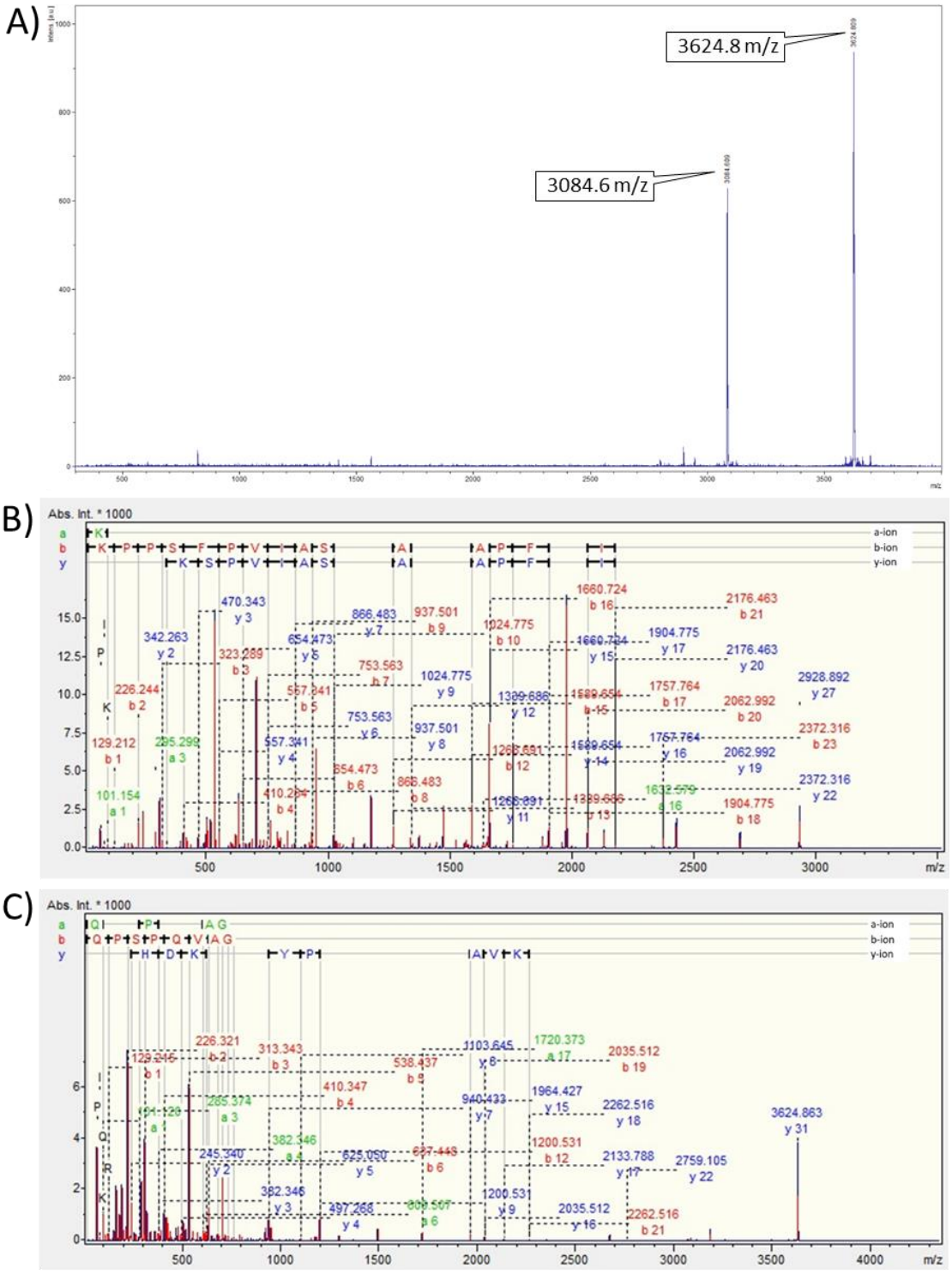


Fig. 21.12. MS spectrum of the parent peaks, m/z 3084.6 and 3624.8 detected in H1-20 (A). MS/MS spectrum of the peaks H1-20 were: m/z 3084.6, β-CN f169-196 [MLPSQPVLSPPQSKVAPFPQPVVPYPQR] (B) and m/z 3624.6, β-CN f169-201 [MLPSQPVLSPPQSKVAPFPQPVVPYPQRDTPVQ] (C), in Table 6.

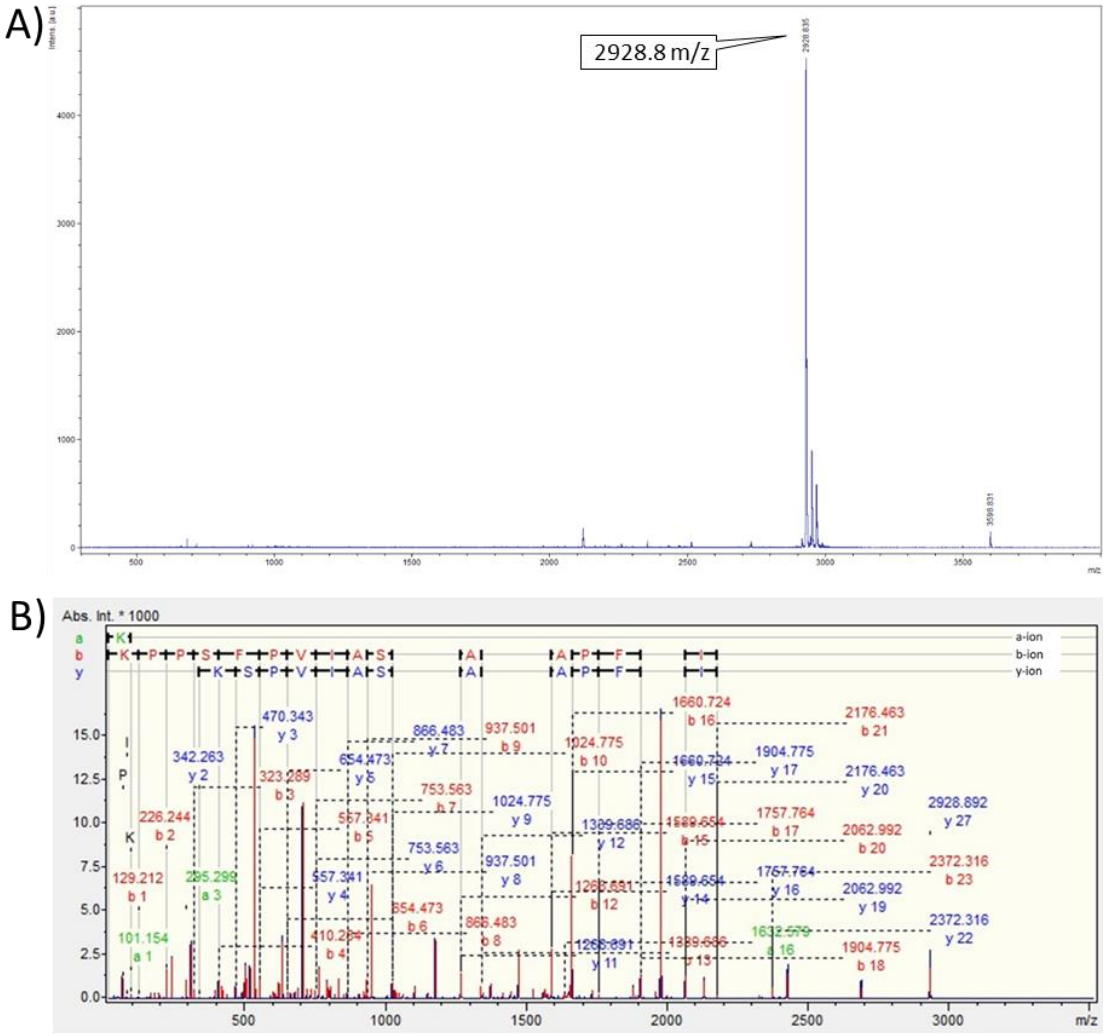


Fig. 21.13. MS spectrum of the parent peak, m/z 2928.8 detected in H1-21(A). MS/MS spectrum of the peak H1-21 was m/z 2928.8, β-CN f169-195 [MLPSQPVLSPPQSKVAPFPQPVVPYPQ] (B) in Table 6.

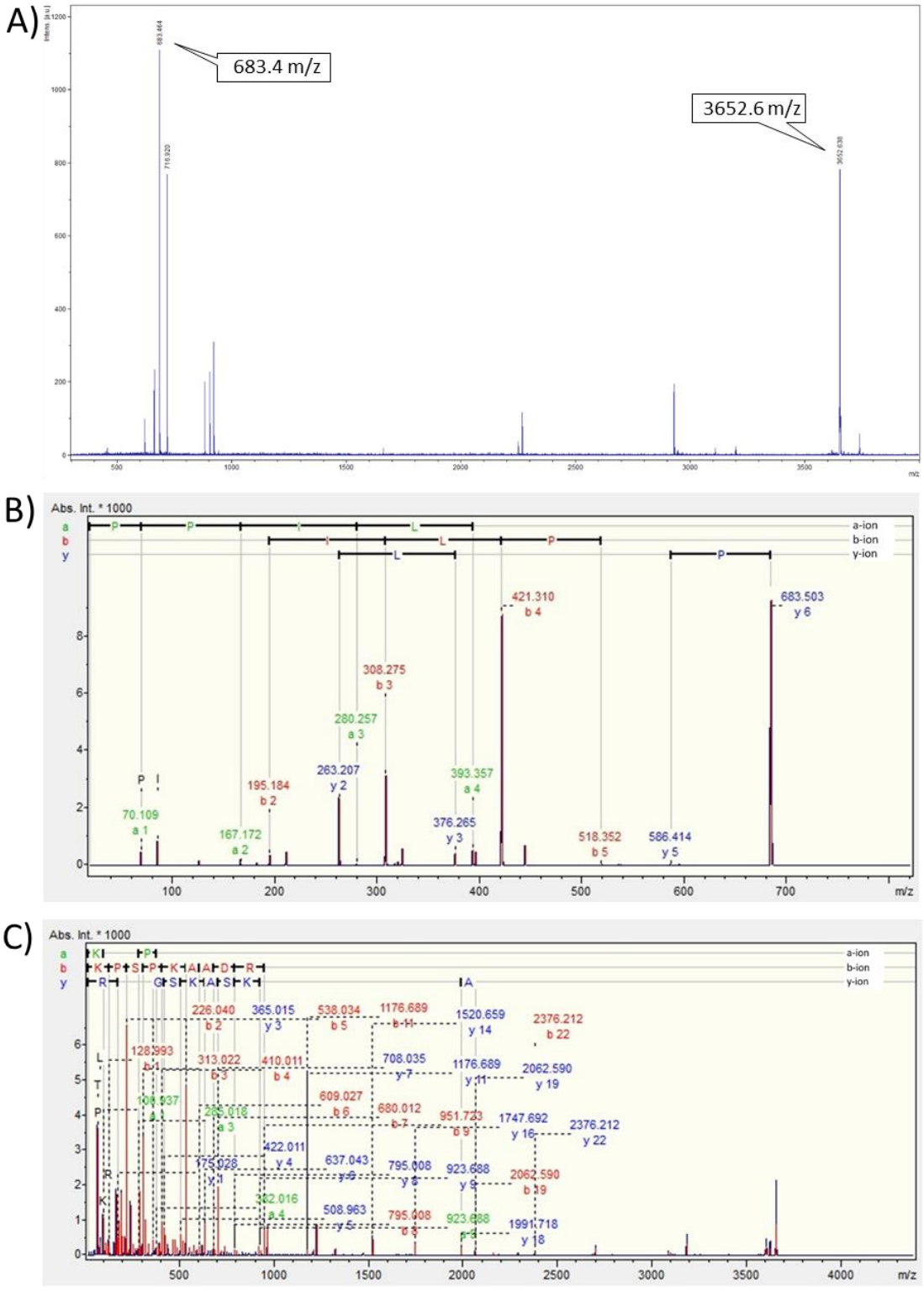


Fig. 21.14. MS spectrum of the parent peaks, m/z 683.4 and 3652.6 detected in H1-22 (A). MS/MS spectrum of the peak H1-22 m/z 683.4 Da for β-CN f95-100 [PPILPF] m/z 3652.6 Da for β-CN f169-201 [MLPSQPVLSPPQSKVAPFPQPVVPYPQQRDTPVQ]

Table 5. Identified peptides in fermented camel's milk.

| Peak ID | Observed mass by MS | Theoretical mass (Expected mass)* | Sequence determined by MS/MS (Sequence expected)* | Origin |
|---|---|---|---|---|
| C3-1 | 597.363 | 596.400 | IRIPV | n.d. |
| C3-2 | 597.321 | 596.400 | IRIPV | n.d. |
| C3-3 | 711.386 | 710.440 | NLRLPV | n.d. |
| | 754.362 | 753.420 | HLLQPF | n.d. |
| | 1021.522 | 1020.520 | $^{76}$RHQNQNPK$^{83}$ | Lactophorin |
| | 1232.775 | n.d. | n.d. | n.d. |
| C3-4 | 1335.659 | n.d. | n.d. | n.d. |
| | 1177.576 | 1176.620 | $^{75}$RRHQNQNPK$^{83}$ | Lactophorin |
| C3-5 | 1098.615 | 1097.470 | $^{65}$TRNEPTEDH$^{73}$ | $\alpha_{s1}$-CN |
| | 1213.633 | 1212.500 | $^{64}$DTRNEPTEDH$^{73}$ | $\alpha_{s1}$-CN |
| | 1798.079 | n.d. | n.d. | n.d. |
| C3-6 | 865.548 | 864.500 | $^{110}$RPRPRPS$^{116}$ | k-CN |
| C3-7 | 865.548 | 864.500 | $^{110}$RPRPRPS$^{116}$ | k-CN |
| C3-8 | 674.370 | 673.350 | $^{221}$HPVPQP$^{226}$ | β-CN |
| | 966.588 | 582.310 | $^{214}$PDPVR$^{218}$ | β-CN |
| | 1309.692 | 1308.580 | NNASHNGNNSAPI | n.d. |
| C3-9 | 759.415 | 758.410 | $^{194}$VPYPQR$^{199}$ | β-CN |
| | 1544.925 | 1543.800 (1543.870) | PPTVERPARNRHD ($^{104}$PPTVERRPRPRPS$^{116}$) | k-CN |
| C3-10 | 929.574 | 928.560 | $^{16}$RPKYPLR$^{22}$ | $\alpha_{s1}$-CN |
| C3-11 | 1019.540 | 1018.500 (1035.530) | HCPVPDPVR ($^{210}$Q$^{**}$EPVPDPVR$^{218}$) | β-CN |
| | 1076.616 | n.d. | n.d. | n.d. |

*expected according to corresponding to sequence of *Camel'sus dromedarius*; ** N-terminal glutamine residue was hydroxylated (-16 Da); C3, fermented camel's milk sample No 3; n.d., not determined; superscipt number indicate the peptide position in the original protein sequnce, peptides without superscript indication was not identified.

Table 6. Identified peptides in fermented mare's milk.

| Peak ID | Observed mass by MS | Theoretical mass (Expected mass)* | Sequence determined by MS/MS (Sequence expected)* | Origin |
|---|---|---|---|---|
| H1-1 | 790.394 | 789.390 | $^{107}$EVSQAKE$^{113}$ | β-CN |
| | 816.432 | 815.420 | $^{55}$REVERQ$^{60}$ | β-CN |
| H1-2 | 951.458 | 950.450 | $^{16}$KHNMEHR$^{22}$ | $\alpha_{s2}$-CN |
| | 1206.619 | n.d. | n.d. | n.d. |
| H1-3 | 823.490 | 822.480 | $^{135}$AIHAQRK$^{141}$ | $\alpha_{s1}$-CN |
| | 927.470 | 944.020 | $^{54}$QREVERQ$^{60}$ (ammonium ion loss from N-terminal) | β-CN |
| | 944.491 | 943.480 | $^{54}$QREVERQ$^{60}$ | β-CN |
| H1-4 | 1129.575 | 1128.570 | $^{45}$KFKHEGQQQ$^{54}$ | β-CN |
| | 1157.581 | 1156.570 | $^{46}$FKHEGQQQR$^{55}$ | β-CN |
| H1-5 | 872.090 | n.d. | n.d. | n.d. |
| | 1241.665 | n.d. | n.d. | n.d. |
| H1-6 | 1129.575 | 1128.570 | $^{45}$KFKHEGQQQ$^{54}$ | β-CN |
| | 1157.581 | 1156.570 | $^{46}$FKHEGQQQR$^{55}$ | β-CN |
| H1-7 | 826.388 | 825.380 | $^{157}$MHQVPQS$^{163}$ | β-CN |
| | 1024.578 | n.d. | n.d. | n.d. |
| H1-8 | 739.356 | 738.350 | $^{157}$MHQVPQ$^{162}$ | β-CN |
| | 786.410 | 785.400 | $^{196}$RDTPVQA$^{202}$ | β-CN |
| | 851.517 | n.d. | n.d. | n.d. |
| | 1396.762 | n.d. | n.d. | n.d. |
| H1-9 | 727.519 | n.d. | n.d. | n.d. |
| | 2062.080 | 797.460 | QGRRGKP | n.d. |
| | 1255.721 | n.d. | n.d. | n.d. |
| H1-10 | 871.484 | 870.470 | $^{65}$RFVQPQP$^{71}$ | β-CN |
| H1-11 | 871.425 | n.d. | n.d. | n.d. |
| H1-12 | 1046.504 | n.d. | n.d. | n.d. |
| | 1232.561 | n.d. | n.d. | n.d. |

Table 6 (continued).

| Peak ID | Observed mass by MS | Theoretical mass (Expected | Sequence determined by MS/MS | Origin |
|---|---|---|---|---|
| H1-13 | 506.406 | n.d. | n.d. | n.d. |
| | 675.222 | 674.280 | $^{74}$QHMPY$^{78}$ | κ-CN |
| | 697.237 | n.d. | n.d. | n.d. |
| | 713.227 | n.d. | n.d. | n.d. |
| | 866.317 | n.d. | n.d. | n.d. |
| | 1050.508 | 1049.510 | $^{49}$EYINELNR$^{56}$ | $\alpha_{s1}$-CN |
| | 1171.640 | n.d. | n.d. | n.d. |
| | 1398.713 | n.d. | n.d. | n.d. |
| | 1980.967 | n.d. | n.d. | n.d. |
| | 2262.040 | n.d. | n.d. | n.d. |
| H1-14 | 785.358 | 784.370 | $^{172}$WFHPAQ$^{177}$ | $\alpha_{s1}$-CN |
| | 805.487 | 804.430 | KVPMPPH | n.d. |
| | 933.512 | 932.470 | $^{196}$RDTPVQAF$^{203}$ | β-CN |
| | 1195.652 | 1267.660 | $^{185}$PFPQPVVPYPQ$^{195}$ | β-CN |
| | 1611.978 | n.d. | n.d. | n.d. |
| | 2067.045 | n.d. | n.d. | n.d. |
| H1-15 | 734.443 | n.d. | n.d. | n.d. |
| | 1021.563 | n.d. | n.d. | n.d. |
| | 1460.719 | n.d. | n.d. | n.d. |
| H1-16 | 1618.056 | n.d. | n.d. | n.d. |
| | 1954.955 | n.d. | n.d. | n.d. |
| H1-17 | 1442.653 | n.d. | n.d. | n.d. |
| | 2069.022 | n.d. | n.d. | n.d. |
| | 2085.990 | n.d. | n.d. | n.d. |
| H1-18 | 1798.163 | n.d. | n.d. | n.d. |
| | 1815.285 | n.d. | n.d. | n.d. |
| H1-19 | 711.451 | 710.440 | $^{145}$NLRLPV$^{150}$ | β-CN |
| | 1797.000 | 1797.070<br>1572.840 | $^{138}$LIPTPNGRS**LRLPVH$^{151}$<br>$^{138}$LNPTNGENLRLPVH$^{151}$ | β-CN |
| H1-20 | 3084.609 | 3083.660 | $^{169}$MLPSQPVLSPPQSKVAPFPQPVPYPQR$^{196}$ | β-CN |
| | 3624.809 | 3623.920 | $^{169}$MLPSQPVLSPPQSKVAPFPQPVPYPQRDTPVQ$^{201}$ | β-CN |
| H1-21 | 2928.835 | 2927.560 | $^{169}$MLPSQPVLSPPQSKVAPFPQPVPYPQ$^{195}$ | β-CN |
| H1-22 | 683.468 | 682.410 | $^{95}$PPILPF$^{100}$ | β-CN |
| | 716.920 | n.d. | n.d. | n.d. |

| | | | |
|---|---|---|---|
| 3652.638 | 3651.951 | $^{169}$MLPSQPVLSPPQSKVAPFPQPVPYPQRDTPVQ$^{201}$ | β-CN |

*expected according to corresponding to sequence of *Equus caballus*; ** serine residue was phophorylated (+79 Da); H1, fermented camel's milk sample No 1; n.d., not determined; superscirpt number indicate the peptide position in the original protein sequnce; peptides without superscript indication was not identified.

# Chapter IV

## Antibacterial activity of Mongolian traditional fermented camel's and mare's milks against enteropathogenic bacteria

### IV-1. Introduction

Mammalian milks act as the primary nutritional source for their newborns. In addition, milk provides a range of functional compounds, including biologically active proteins beyond its basic nutrients. Once milk proteins degraded, they release peptide fragments that have a range of biological properties that can be different from those of the original protein (Dallas *et al*, 2015). These functional milk peptides are originated from both casein, including $\alpha_{s1}$-, $\alpha_{s2}$-, β-, and κ-casein and whey proteins, including α-lactalbumin, β-lactoglobulin, and lactoferrin (Dallas *et al*, 2015). They can be released from milk proteins by native proteases (Dallas *et al*, 2015), during production process such as fermentation and during digestion (Meisel & Bockelmann, 1999; Korhonen & Pihlanto, 2006). These peptides have a range of activity, including antibacterial (Mohanty *et al*, 2014), antioxidant (Kudoh *et al*, 2001), dipeptidyl peptidase IV (DPP-IV) inhibition (Uchida *et al*, 2011), angiotensin converting enzyme (ACE) inhibition (Maeno *et al*, 1996), mineral binding (Hansen *et al*, 1996) and immuno-modulating functions (Nielsen *et al*, 2017).

Antimicrobial bioactive peptides or fractions can be inhibited the growth of Gram-positive and -negative microorganisms. For instance, Antimicrobial peptides such as isracidin and lactoferricin, have been broadly studied *in vivo* and *in vitro*. The first antimicrobial peptide derived from $\alpha_{s1}$-CN was isracidin, which was released by the action of chymosin (Hill *et al*, 1974; Lopez-Exposito & Recio, 2008). Isracidin has been shown *in vivo* protection activity against an array of pathogens including *Listeria monocytogenes* and *Staphylococcus aureus* in mice and against *S. aureus* in rabbits, guinea pigs, and sheep (Lopez-Exposito & Recio, 2008). The peptide also exhibited *in vitro* inhibitory effect on the growth of lactobacilli and other Gram-positive bacteria at high concentrations (0.1-1 mg/mL) (Hill *et al*, 1974; Lopez-Exposito & Recio, 2008).

Lactoferricin, a bioactive peptide derived from lactoferrin, has been shown to exhibit anti-fungi, anti-parasite and antimicrobial activity against both Gram-positive and -negative bacteria (Bellamy *et al*. 1992; Lopez-Exposito & Recio, 2008). Several *in vivo* studies have been conducted to check the protective effects of lactoferricin against *S. aureus* and infections caused by *Toxoplasma gondii* have been reported (Bellamy *et al*, 1992; Tanaka *et al*, 1995; Wakabayashi *et al*, 1996; Isamida *et al*, 1998; Recio & Visser, 1999a). Furthermore, lactoferrampin, which is also derived from lactoferrin, has been reported to show antibacterial activity toward *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (Van Der Kraan *et al*, 2004; Lopez-Exposito & Recio, 2008). Several peptide fragments derived from bovine α-lactalbumin and β-lactoglobulin digested by tryptic or chymotryptic action have been revealed to show moderate antimicrobial activity against Gram-positive bacteria (Pellegrini *et al*, 1999; Pellegrini *et al*, 2001; Lopez-Exposito & Recio, 2008). In addition, bovine $\alpha_{s2}$-CN f(183-207) peptide and f(164-179) peptide hydrolysed by pepsin have shown inhibitory effects on a broad spectrum of both Gram-positive and -negative pathogens (Recio & Visser, 1999a; Lopez-Exposito & Recio, 2008). It has been reporte that peptides derived from $\alpha_{s2}$-CN f(164-207) also exhibited inhibitory effects of *Listeria innocua* (Mccann *et al*, 2006). The antimicrobial activity and its mechanisms have been clarified by several researchers (Deegan *et al*, 2006; Lopez-Exposito *et al*, 2007; Umuhumuza *et al*, 2011). Antimicrobial peptides are known mostly to interact with bacterial cell wall or membrane to exhibit their antimicrobial activities, for example cationic peptides interact with anionic phospholipids and lipopolysaccharides components in cell walls and membranes, leading to form a pore and desturbance of electro potential difference outer and inner membrane of bacterial cells.

The health benefits of camel's and horse's milks are mainly due to the presence of bioactive peptides that can be liberated from milk proteins during fermentation by the proteolytic activity of lactic acid bacterial enzymes (El-Agamy *et al*, 1992; Ganzorig *et al*, 2016). Fermented camel's milk shows inhibitory activity against Gram-positive and -negative bacteria, including *E. coli, L. monocytogenes, S. aureus,* and *Salmonella* Typhimurium (Benkerroum *et al*, 2004). Well known inhibitory compounds detected in fermented camel's milk are lysozyme, lactoperoxidase, lactoferrin, hydrogen peroxide, and immune proteins (Cagno, 2002). In addition, fermented

camel's milk contains various bioactive peptides that are released from the parent proteins during the fermentation process, and the enzymes activity of starter culture (El-Agamy *et al*, 2009; Meisel 2001; Clare & Swaisgood, 2000). Up to date, there are lack of studies conducted on Mongolian fermented camel's and mare's milks, and their antimicrobial activities involved with presence of bioactive peptides. Fermented camel's and mare's milks made at home without a standardized fermentation process are consumed under non-ideal hygiene conditions by many of the nomadic and local people in Mongolia (Bayarsaikhan *et al*, 2011). Therefore, the aim of current study is to evaluate the antimicrobial activity of Mongolian fermented camel's and mare's milks using membrane separation techniques, aiming to isolate bioactive peptides as the major components.

## IV-2. Materials and methods

### IV-2-1. Sample preparation for antimicrobial activity assay

Fermented milks (100 mL) were warmed to 35°C and defatted by centrifugation at 500 x *g* for 10 min at 20°C. The fat layer was removed and then supernatant as a whey was centrifuged again at the same condition to remove residual fat and cells. The caseins and insoluble compounds were removed by centrifugation at 39800 x *g* for 60 min at 4 °C. The supernatant, namely acid whey, was sequentially ultra-filtered through a Centriprep ultra-filtration unit (Amicon, Millipore, USA) with different molecular weight cut-off membrane (Fig. 22). In brief, the whole whey was first ultra-filtered using Centriprep YM-10 with nominal molecular weight limit (NMWL) of 10 kDa by centrifugation at 3000 x *g* for 30 min at 4 °C. This step yielded two fractions: retentate (>10 kDa; F1) and permeate (<10 kDa). A permeate was further ultra-filtered by Centriprep YM-3 (NMWL 3,000) to obtain second retentate (3-10 kDa; F2) and permeate (<3 kDa; F3). All retentates and permeates were lyophilized and dissolved in 0.1% of trifluoracetic acid. Sample solutions were then sterilized by a 0.2 μm filter and stored at -20 °C till further experiments performed.

### IV-2-2. Preparation of nisin solution

Ten milligram of nisin from *Lactococcus lactis* (Sigma-Aldrich, Tokyo, Japan) was dissolved into 1 mL of nisin buffer (0.02 N HCl, 0.75% NaCl solution) to obtained 10000 International Unit (IU)/mL concentration. And then nisin solution was sterilized by a 0.2-μm filter membrane (Advantec), and further obtained serial dilution such as 1000, 100, 10, and 1 UI/mL using the same buffer. Nisin solution was freshly prepared on the day of antimicrobial assay conducted. For the selection of optimum concentration of nisin on inhibiting the growth of pathogenic bacteria, the antimicrobial activity of nisin was assessed by disc diffusion method against all of pathogenic bacteria.

**IV-2-3. Preparation of ampicillin solution**

Ten milligram of ampicilin was dissolved into 10 mL of milli-Q water to obtain 1 mg/mL concentration. After filter-sterilization by a 0.2 μm filter membrane (Advantec), ampicillin solution was diluted to carry out 0.1 mg/mL concentration, and then divided into 500-μL aliquots and kept at -20˚C until used.

**IV-2-4. Antimicrobial activity assay**

The disc-diffusion assay was used to evaluate the antimicrobial activity of retentate and permeate isolated from fermented camel's and mare's milks whey. Lyophilized retentate and permeate, dried powders were dissolved in 0.1% TFA at concentration of 100 mg/mL and sterilized using 0.2 μm cellulose acetate membrane filters (Advantec, Tokyo, Japan). Paper disc for antibiotic assay (hereafter as discs) with 6 mm diameter (Advantec, Tokyo, Japan) were placed into sterile Petri dishes and impregnated with 30 μL of the samples, including controls. The discs were allowed to dry at room temperature for 1 h. The 1.2% (w/v) agar mixed with Brain heart infusion or Luria-Bertani medium pre-sterilized and incubated at 50°C was thoroughly mixed with an overnight culture of each pathogenic bacteria ($10^7$-$10^9$ CFU/mL), including *S.* Typhimurium LT-2, *Shigella sonnei*, methicillin-resistant *Staphylacoccus aureus* (MRSA), methicillin-sensitive *Staphylacoccus aureus* (MSSA), *L. monocytogenes* and *E. coli* O157, poured into the Petri dishes, and incubated at room temperature for 30 min. The discs were then placed on the pathogen seeded BHI or LB

agar plates. These were first incubated at 4°C for 1 h to allow antimicrobial compounds to be diffused into the agar, then further incubated at 37°C for 24 h aerobically. Antimicrobial activities were evaluated by measuring diameters of growth inhibition zones around the discs in millimeters. Discs impregnated with 0.1% Trifluroacetic acid and milli-Q water were used for controls. As positive controls, solutions of 0.1 mg/mL of ampicillin and 10000 IU/mL of nisin from *L. lactis* (Merck KGaA) were used against Gram-negative and -positive bacteria, respectively.

**IV-2-5. Peptide fractionation by RP-HPLC**

Whey ultra-filtrates whose molecular weight less than <3 kDa, from C3 (fermented camel's milk) and H1 (fermented mare's milk) separated by RP-HPLC to isolate peptide fractions. The experimental condition of RP-HPLC method was totally same, described in Chapter 3 (see section II-2-3) of this study. In total, 11 peaks were collected from C3 (C3, Fig. 25) sample by manually while 12 fraction collected from H1 (H1, Fig. 25) sample using time fractionation for every 5 min. Each chromatographic fraction was finally lyophilized.

**IV-2-6. Statistical analysis**

Data were expressed as means ± standard deviations (SD) from three replications. The statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Data were considered significant at *P* value less than 0.05.

**IV-3. Results**

Research focus in this chapter was on the fractionation of whey peptides from fermented camel's and mare's milks and to evaluate their antimicrobial activity. The wheys from fermented camel's and mare's milks were ultra-filtered to obtain the fractions with different molecular weight, namely F1: >10 kDa, F2: 3-10 kDa and F3: <3 kDa (Fig. 22). These fractions were *in vitro* evaluated for antimicrobial activity using agar diffusion assay. The agar diffusion assay is suitable method to check the antimicrobial activity of food antimicrobials, peptides and hydrolysates. The

efficacy of fermented milk whey fractions its antibacterial activity were measured by inhibition zone (mm) and values were summarized in Table 7.

An each of 3 samples from Mongolian camel's and mare's fermented milks and 3 different fractions (<3 kDa, 3-10 kDa and >10 kDa) were obtained from each sample. In total, antimicrobial activities of 18 fractions were evaluated against Gram-positive (*L. monocytogenes* and *S. aureus* species) and Gram-negative (*E. coli, S. sonnei* and *S.* Typhimurium) bacteria. As a positive control, ampicillin exhibited antimicrobial activity to all indicator pathogens (11.3 ± 0.9 to 38.2 ± 11.1 mm) other than MRSA which is resistant to several antibiotics. On the other hand, the nisin derived from *L. lactis* was selectively inhibited only the growth of Gram-positive bacteria (10.7 ± 0.5 to 18.7 ± 1.4 mm), such as *L. monocytogenes* and *S. aureus* species. As a negative control, milli-Q water and 0.1% TFA taken as a dissolvent for all fraction did not show any inhibition. In general, fraction F2 and F3 from both fermented milk showed antimicrobial activity only against Gram-negative microorganisms, *S. sonnei* (Fig. 23) and *S.* Typhimurium (Fig. 24) to different extents. However, fractions from all sample did not show any inhibition against MRSA, MSSA, *E.coli* and *L. monocytogenes*, while positive controls, ampicillin (11.3-38.2 mm) and nisin (11.1-18.7 mm) were successfully inhibited (Table 7). Among the camel's fermented milk (CFM) samples, fractions F2 and F3 from CFM2 showed the antimicrobial activity against *S. sonnei* with values of 8.6 ± 1.1 and 9.6 ± 0.5 mm, respectively (Table 7), while fractions from CFM1 and CFM3 were not displayed any inhibition. For the mare's fermented milk samples, fractions F2 and F3 from both HFM1 and HFM2 were exhibited the inhibition activity with comparable level to each other. With regard to the antibacterial activities of obtained fractions, F2 and F3 from CFM2, CFM3, HFM1 and HFM2 showed the inhibition activity toward *S.* Typhimurium, while fractions from CFM1 and HFM3 exhibited no inhibition (Table 7). In general, fractions extracted from HFM shows higher inhibition activity against both *S.* Typhimurium and *S. sonnei* comparing to fermented camel's milk sample.

To explore the antimicrobial activity of the F3 fractions from CFM3 and HFM1, ultra-filtrates <3 kDa were further separated by RP-HPLC (Fig. 25). The peptides <3 kDa were eluted into 11 peaks for CFM3 and 12 fractions for HFM1. In total, antibacterial activity of 23 fractions

were evaluated against six enterophatogenic bacteria under the same method described in this chapter. (see section IV-2-4). According to result of the first trial, no antibacterial activity found yet among these 23 peptide fractions. It is assumed that peptide concentration in the RP-HPLC fraction was not enough to inhibit the growth of pathogenic bacteria.

**IV-4. Discussion**

Ampicillin is an antibiotic in which the β-lactam ring present, consisting an amino group in the structure which reacts with the active site of an enzyme, transpeptidase (Mohanty *et al*, 2016). It is known to be responsible for construction of cell wall in bacteria. In this context, ampicillin causes breakdown of the cell wall by stopping the biosynthesis of peptidoglycan and finally leads to death of bacteria (Ghooi & Thatte, 1995). But some bacteria, for example *S. aureus* is resistant to ampicillin because of secreting the β-lactamases which catalyzes a hydrolytic reaction of the β-lactam ring of ampicillin (Cha *et al,* 2008). Nisin is one of antimicrobial agent produced by *L. lactis* and active to inhibit the growth of Gram-positive bacteria. Antimicrobial substances are supposed enter to cells via their outer membrane pore and reacts with intracellular enzymes (Russell & Diez-Gonzalez, 1998). But nisin cannot penetrate the outer membrane of Gram-negative bacterial cell wall, because of its high molecular weight, approximately 4600 Da, hence it is only effective to Gram-positive bacteria (Brötza & Sahl, 2000).

It is well known that most bioactive peptides are inactive when they exist in the original protein sequence but can be activated by the action of proteolytic enzymes during either fermentation process of dairy foods or digestion in the human gastrointestinal tract (Korhonen & Pihlanto, 2006). Many bacteriocins generated by various bacterial strains, including LAB have been reported to date. For example, Arakawa *et al*, (2016) found a bacteriocin-like substance with molecular weight of 2.6-3.0 kDa, produced by LAB strain isolated from Mongolian *airag*. Wulijideligen *et al*, (2012) also found another bacteriocin with different molecular size about 2 kDa, produced by LAB strain, *Leu.mesenteroides* 406 from Mongolian *airag*. Also, higher molecular weight bacteriocins has been reported by Batdorj *et al* (2006) and they separated two bacteriocins whose molecular weight 5206 and 5218 Da from Mongolian airag. Those findings

support the result of this study that peptide fractions F3 (<3 kDa) and F2 (3-10 kDa) isolated from fermented camel's and mare's milks are likely contain bacteriocin-like substances.

Antimicrobial activity of whey fractions obtained in this study were found against only Gram-negative bacteria, *S.* Typhimurium and *S. sonnei.* Similar findings were reported in some previous studies. For instance, Alhaj *et al,* (2018) observed that water soluble permeate (<3kDa), isolated from camel's fermented milk showed the antimicrobial activity to *S.* Typhimurium. The peptides isolated from camel's milk fermented by *L. plantarum* inhibited the growth of Gram-positive and -negative bacteria (Muhialdin & Algboory, 2018). Ultra-filtrated peptide fractions of camel's milk casein hydrolysate were exhibited the antibacterial activity to Gram-negative bacteria (Kumar *et al*, 2016). The action between antimicrobial peptides and Gram-negative bacteria related to cationic antimicrobial peptides which contain a high amount of hydrophobic amino acids (Yount & Yeaman, 2013). Therefore, it was considered that the adsorption of antibacterial peptides to the cell surface could be as an initial step in the interaction between the peptides in the fractions and bacterial cell. This interaction happens between the cationic peptide and negatively charged substance present in the bacterial cell wall, such as phosphate groups within the outer membrane of Gram-negative bacteria. Once they connected with cell membrane could enter into cell body stimulated by hydrophobic interaction (Jenssen *et al,* 2006) and antibacterial peptides the cause destruction and permeabilisation of the cytoplasmic membrane.

From this study, it can be concluded that ultra-filtration technique could be used to obtain the different molecular weight range peptides from fermented camel's and mare's wheys and to evaluate its activity. However, it was observed that lower molecular weight fractions which is lower than <10 kDa, exhibited antimicrobial activity against Gram-negative pathogens while higher molecular weight fraction showed no activity. This may be due to higher concentration of antibacterial substances are exist in the lower molecular weight fractions compared to higher one. To achieve the enough yield of antibacterial substances, as being peptides most conceivable judging from the literatures, further purification using an ion-exchange chromatography should be examined. Application of antibacterial substances present in beverages such as traditional fermented milk products is highly potential to be an alternative for the usage of antibiotics, because

overuse, inappropriate consumption and application of antibiotics have driven an emergence of multidrug-resistant pathogens. This trend has nowadays become a global concern to be solved as soon as possible (Medina & Pieper, 2016).

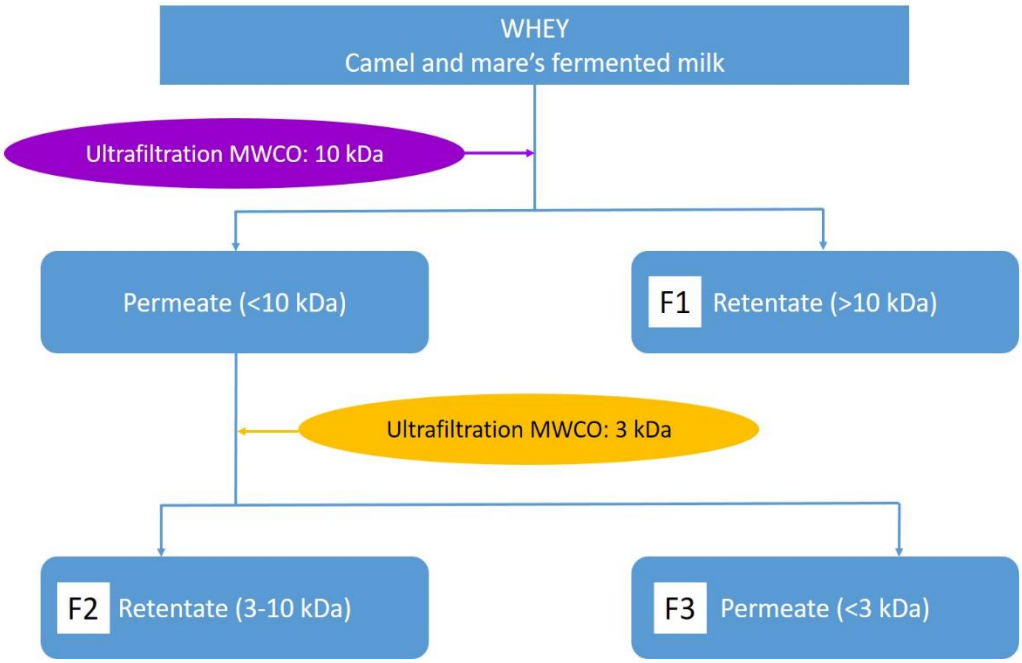


Fig. 22. Flow chart of sequential ultrafiltration protocol of camel's and mare's fermented milk whey using different membrane cut-off size. F1, fraction 1 contain whey >10 kDa; F2, fraction 2 contain whey 3-10 kDa; F3, fraction 3 contain whey <3 kDa. MWCO, molecular weight cut-off.

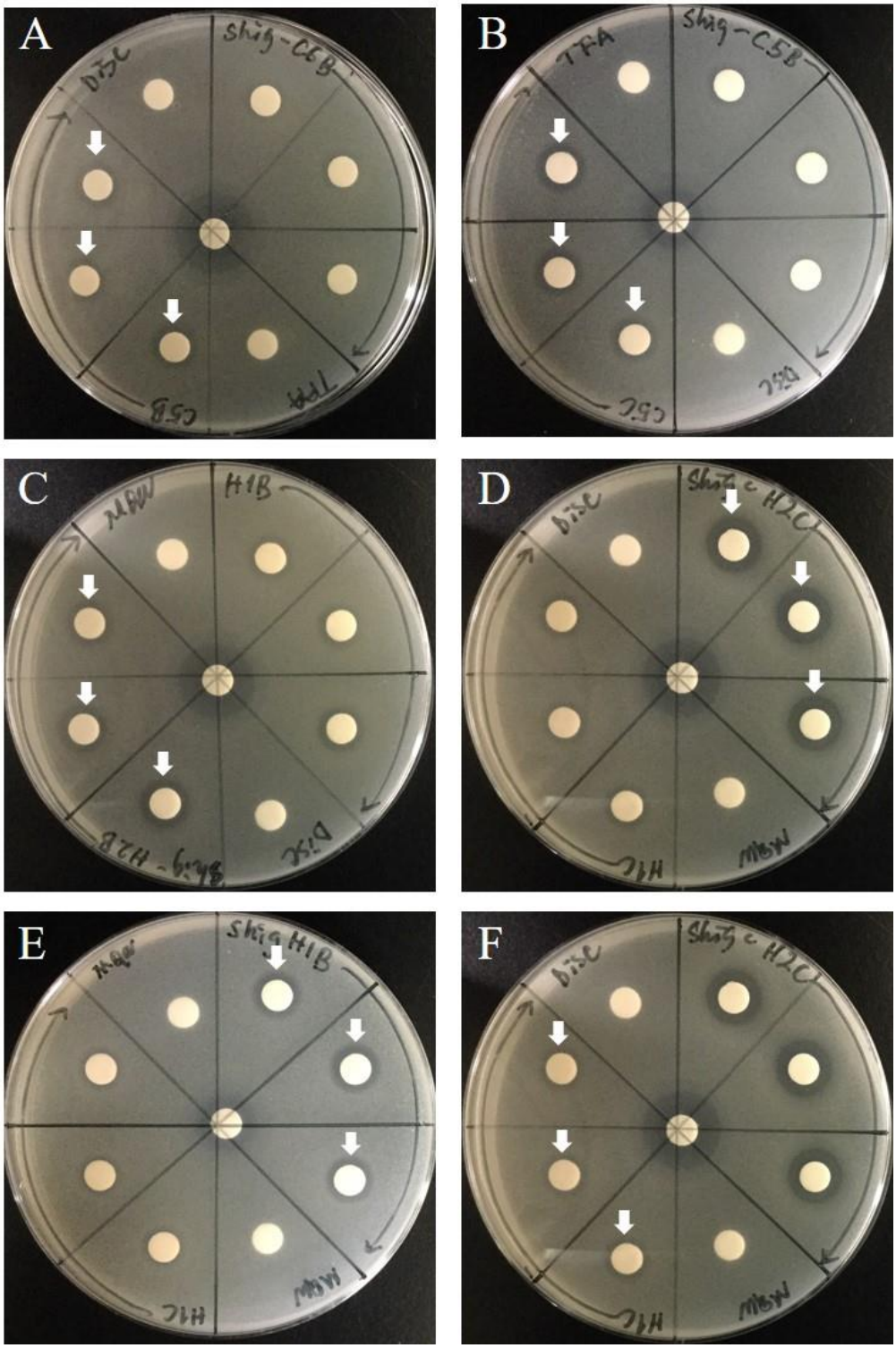


Fig 23. Antibacterial activity of fraction from CFM2 (F2 in panel A and F3 in panel B), HFM1 (F2 in panel C and F3 in panel D) and HFM2 (F2 in panel E and F3 in panel F) against *Shigella sonnei.* In each panel, triplication of individual fraction showed the inhibition activity was indicated by a white arrowhead. Three individual experiments had shown the similar results; therefore, the representative data was indicated in this figure.

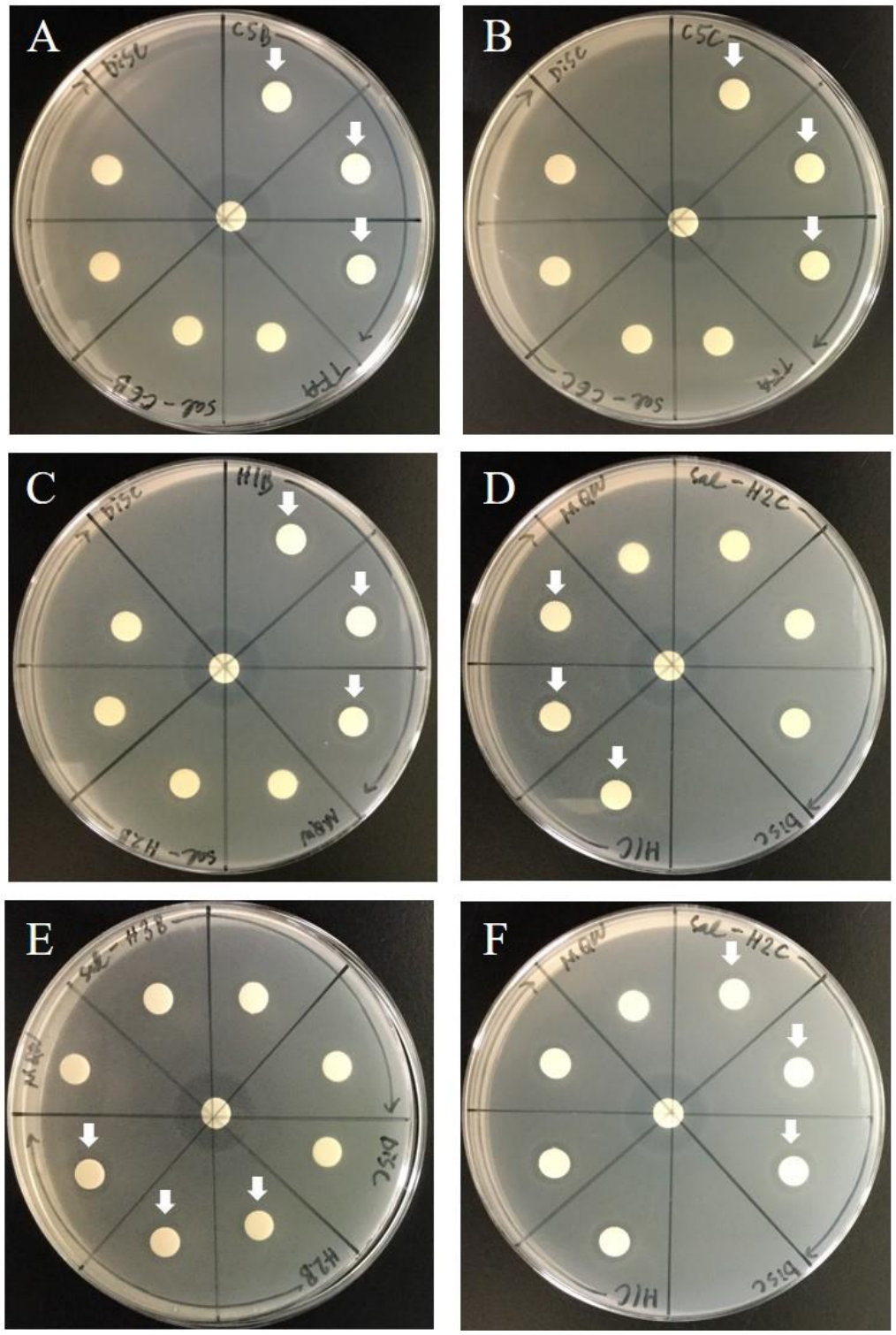


Fig 24. Antibacterial activity of fraction from CFM2 (F2 in panel A and F3 in panel B), HFM1 (F2 in panel C and F3 in panel D) and HFM2 (F2 in panel E and F3 in panel F) against *Salmonella Typhimurium.* In each panel, triplication of individual fraction showed the inhibition activity was indicated by a white arrowhead. Three individual experiments had shown the similar results; therefore, the representative data was indicated in this figure.

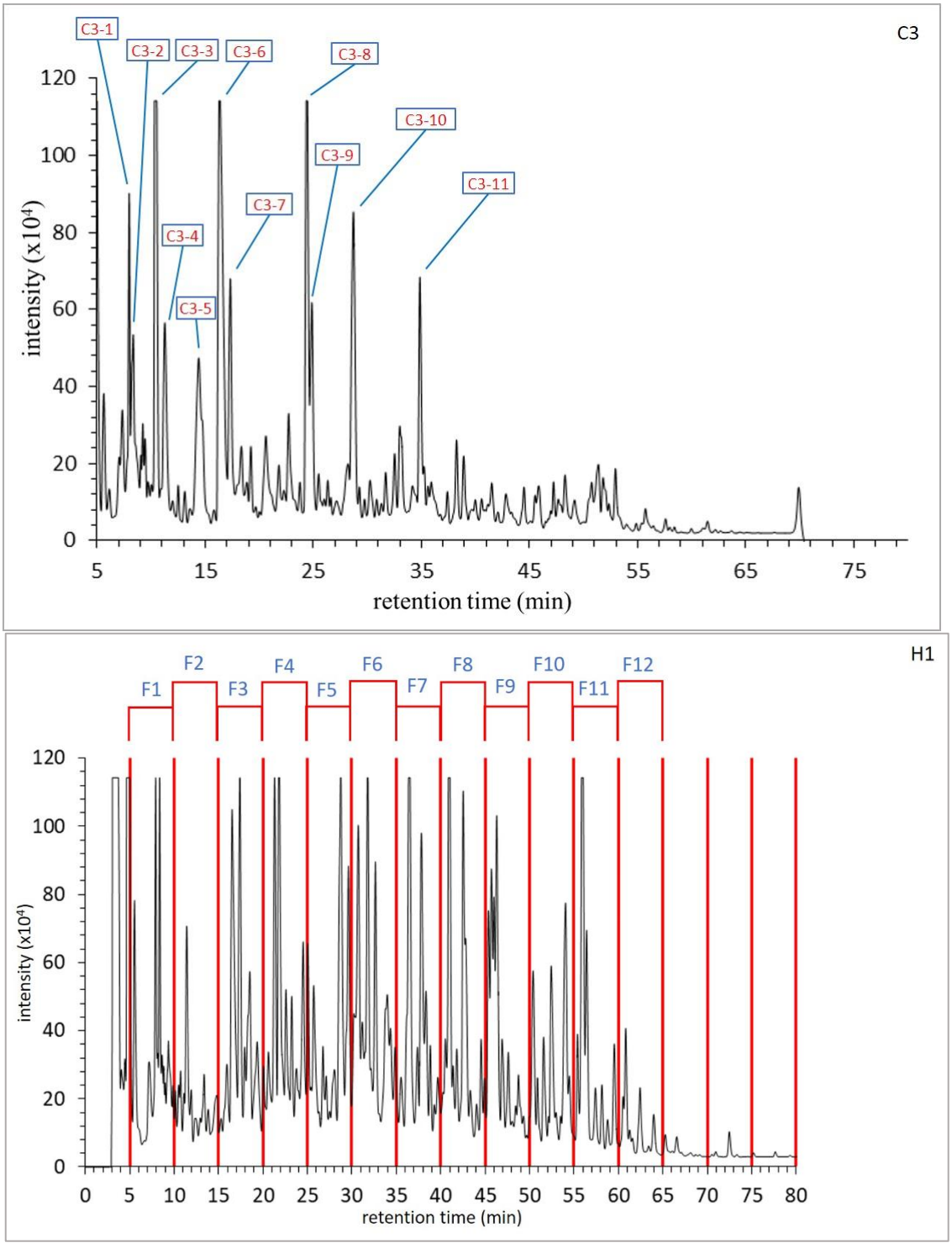


Fig. 25. RP-HPLC separation of <3 kDa peptides extracted from (C3) fermented camel's milk and (H1) fermented mare's milk, F1-F12; fractions collected.

Table 7. Antimicrobial activity (zone of inhibition in mm) of peptide fractions from fermented camel's and mare's milk whey.

| whey fraction | CFM | | | HFM | | | Control | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | Ampicillin | Nisin |
| | | | | *MRSA* | | | | |
| F1 | NI | NI | NI | NI | NI | NI | NI | 11.1 ± 2.1 |
| F2 | NI | NI | NI | NI | NI | NI | | |
| F3 | NI | NI | NI | NI | NI | NI | | |
| | | | | *MSSA* | | | | |
| F1 | NI | NI | NI | NI | NI | NI | 38.2 ± 1.1 | 18.7 ± 1.4 |
| F2 | NI | NI | NI | NI | NI | NI | | |
| F3 | NI | NI | NI | NI | NI | NI | | |
| | | | | *E.coli* | | | | |
| F1 | NI | NI | NI | NI | NI | NI | 11.3 ± 0.9 | NI |
| F2 | NI | NI | NI | NI | NI | NI | | |
| F3 | NI | NI | NI | NI | NI | NI | | |
| | | | | *L. monocytogenes* | | | | |
| F1 | NI | NI | NI | NI | NI | NI | 30.4 ± 1.5 | 10.7 ± 0.5 |
| F2 | NI | NI | NI | NI | NI | NI | | |
| F3 | NI | NI | NI | NI | NI | NI | | |
| | | | | *S. sonnei* | | | | |
| F1 | NI | NI | NI | NI | NI | NI | 14.9 ± 0.9$^{b}$ | NI |
| F2 | NI | 8.6 ± 1.1$^{Aa}$ | NI | 9.2 ± 1.4$^{Aa}$ | 9.6 ± 0.5$^{Aa}$ | NI | | |
| F3 | NI | 9.6 ± 0.5$^{Aa}$ | NI | 9.9 ± 0.8$^{Aa}$ | 10.9 ± 0.3$^{Aa}$ | NI | | |
| | | | | *S.* typhimurium | | | | |
| F1 | NI | NI | NI | NI | NI | NI | 17.3 ± 0.4$^{b}$ | NI |
| F2 | NI | 8.7 ± 0.7$^{Aa}$ | 7.6 ± 0.8$^{Aa}$ | 9.7 ± 0.5$^{Aab}$ | 9.6 ± 1.2$^{Aab}$ | NI | | |
| F3 | NI | 9.4 ± 0.7$^{Aa}$ | 7.8 ± 1.2$^{Aa}$ | 9.1 ± 0.6$^{Aa}$ | 10.4 ± 0.5$^{Aab}$ | NI | | |

The antimicrobial activities were assessed by measuring the diameter of growth inhibition zones around the discs as mean ± SD from three independent experiments (n=9). Different superscript lowercase letters in the same row represent significant differences ($p$<0.05) of antimicrobial activity among type of fermented milk, its samples and nisin to pathogenic bacteria while same superscripts uppercase letters in the same column do not differ significantly ($p$<0.05) among the fractions. CFM: camel's fermented milk; HFM: horse fermented milk; NI: no inhibition; F1: fraction having peptide size (MW) in the range of >10 kDa; F2: fraction having peptide size (MW) in the range of 3-10 kDa and F3 having peptide size (MW) in the range of <3 kDa.

# Chapter V

## General discussion

The first objective of this study was to search novel milk oligosaccharides from addax, a Bovidae species. Several sialyl oligosaccharides, including a novel UDP-sialyl *N*-acetyllactosamines, Neu5Gc(α2-6)Gal(β1-4)GlcNAcα1-UDP, have been identified in addax colostrum. In addition, UDP-Gal, UDP-GlcNAc and UDP-hexose were also found. Therefore, addax colostrum can be a good source of acidic oligosaccharides. It might be necessary to clarify that in which species milk and colostrum contain UDP sialyl *N*-acetyllactosamine or UDP *N*-acetyllactosamine, among closely related species of Bovidae such as cows, water buffalo, buffalo as well as some Antelopinae in the future. It was unsuccessful to conduct the exploration of bioactivity of the UDP-oligosaccharides found in this study due to their low yields. In future, *in vitro* studies on prebiotic activity of UDP- oligosaccharides to colonic epithelial cells, some bifidobacterial strains and pathogenic bacterial strains shuld be performed.

The identified peptides in fermented camel's milk in this study were derived from β-casein, $\alpha_{s1}$-casein, lactophorin and κ-casein, while peptides in fermented mare's milk were derived from β-casein, $\alpha_{s1}$-casein, $\alpha_{s2}$-casein and κ-casein. Original proteins of the found peptides were the major components of camel's and mare's milks. Low molecular weight fractions of the fermented milks, whose molecular weight lower than <10 kDa, inhibited the growth of Gram-negative pathogenic bacteria while higher molecular weight fraction did not show any activities. It was speculated that antimicrobial activity may stem from a presence of cationic peptides, usually interact with Gram-negative bacteria. Antimicrobial activity was not observed in RP-HPLC fractions obtained from ultra-filtrate (<3 kDa) of both fermented milks, probably due to low amount of peptides in those fractions. Therefore, it is considerable to modify the experimental design on peptide separation such as using an ion-exchange chromatography or other proteomic tools to find the antimicrobial activity substances from ultra-filtrates of fermented camel's and mare's milks.

## General conclusion

First, addax colostrum was found to contain UDP-oligosaccharides and potentially biofuntional sialyl oligosaccharides that are not reported in milks of livestock animals except for ovine. Second, fermented camel's and mare's milks contain an array of peptides derived from different proteins as compared to raw milks of domestic animals. Third, it was found that fermented camel's and mare's milks are good sources of antimicrobial substances. In future, biological significance of UDP-oligosaccharides, sialyl oligosaccharides and peptides derived from fermented milks should be clarified. All in all, raw milks from wild animals and fermented products made by livestock animals' milks are highly potential as sources of novel bioactive substances.

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## 和文要旨

本研究は乳中の機能性成分探索を目的とし、ウシ科に属する野生動物（アダックス）の乳中ミルクオリゴ糖プロファイル解析、モンゴル産発酵ラクダ乳および発酵馬乳に含まれるペプチドプロファイル解析を行い、発酵乳由来低分子化合物について抗菌活性のスクリーニングを行った。

哺乳類が分泌する常乳や初乳はラクトースを主成分とするミルクオリゴ糖を含む。ウシ、ヒツジ、ヤギといったウシ科動物のミルクオリゴ糖は既に解析されているが、ウシ科ブラックバック亜科に属するアダックスは未解析であった。本研究では、$^{1}$H-NMR の手法によりアダックス初乳中のミルクオリゴ糖プロファイル解析を行った。その結果、数種の酸性オリゴ糖 Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc (ジシアリルラクトース)、Neu5Gc(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc (ジグリコリルラクトース)、Neu5Ac(α2-3)Gal(β1-4)Glc (3'-シアリルラクトース)、Neu5Ac(α2-6)Gal(β1-4)GlcNAc (6'-シアリル-N-アセチルラクトサミン)、Neu5Gc(α2-3)Gal(β1-4)Glc (3'-グリコリルラクトース)、Neu5Gc(α2-6)Gal(β1-4)Glc (6'-シアリルラクトース)、Neu5Gc(α2-6)Gal(β1-4)GlcNAc (6'-グリコリルラクトサミン) を検出した。さらに、オリゴ糖ヌクレオチド Neu5Gc(α2-6)Gal(β1-4)GlcNAcα1-UDP (ウリジン 5'-ジフォスフォ-N-アセチルラクトサミン) を新規に見出した。アダックスの初乳中酸性オリゴ糖プロファイルと他のウシ科 由来酸性オリゴ糖プロファイルを比較した結果、新規に見出したオリゴ糖ヌクレオチドの生理機能は不明である。UDP-*N*-アセチルラクトサミンをドナーとして認識する糖転移酵素の存在が示唆された。

発酵ラクダ乳と発酵馬乳はモンゴルで広く消費されており、効率的な栄養源かつ健康の源と考えられている。その成分の中で、ヒトの健康機能性を示す候補との一つとしてペプチドが挙げられる。乳タンパク質は発酵過程で微生物由来タンパク質分解酵素により分解され多くの生理活性ペプチドを遊離し、その結果発酵乳の健康機能性を強化する。本研究では、主としてモンゴル南部で採集した発酵ラクダ乳と発酵馬乳を試料として用いた。発酵乳試料からホエイを調製し、分画分子量 3 および 10 kDa の限外濾過膜を用いて分画した。これらを用い、ディスクディフュージョンアッセイ法により抗菌活性を見積もった。その結果、両発酵乳から得た主として分子量 10 kDa 以下の画分にグラム陰性菌 *S.* Typhimurium および *S. sonnei* に対する抗菌活性を見出した。

抗菌活性を示した分画分子量 10 kDa の限外濾過膜を透過した濾液を RP-HPLC により分離し、MALDI-TOF MS/MS を用いて同定した。その結果、発酵ラクダ乳から 11 種、発酵馬乳から 24 種のペプチドを同定した。発酵ラクダ乳に見出したペプチドのうち 4 種は β-カゼイン由来、3 種は $\alpha_{s1}$-カゼイン由来、2 種は κ-カゼインおよびラクトフォリン（PP3）由来であった。発酵馬乳に見出したペプチドのうち、19 種は β-CN 由来、3 種は $\alpha_{s1}$-カゼイン由来、1 種は κ-カゼインおよび $\alpha_{s2}$-カゼイン由来であった。HPLC により分画した結果得られた 23 の画分について既述の抗菌活性試験を試みたが、いずれの画分についても抗菌活性を見出すことはできなかった。これは、RP-HPLC により得られた各分に含まれるペプチド量が少なく、抗菌活性を検出するために必要な閾値未満であったためと考えられた。先行研究で $\alpha_{s1}$-カゼイン f16-22 [RPKYPLR]、β-カゼイン f194-199 [VPYPQR]、f210-218 [QEPVPDPVR]、f221-226 [HPVPQP]が *E. coli*、 *S. aureus*、*S. faecalis*、*S. dysenteria* に対し抗菌活性を示すことが報告されており、本研究で見出されたペプチドも同様の活性を有することが示唆された。

本研究の結果から、野生動物の乳や家畜発酵乳は、糖質やペプチドなど新規機能性成分の良い単離源になり得ると結論付けた。