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Isolation and identification of novel casein-derived bioactive peptides and potential functions in fermented casein with *Lactobacillus helveticus*

Mengzhu Fan[a], Tingting Guo[a], Wanru Li[a], Jing Chen[a], Fushuo Li[a], Chao Wang[a], Yi Shi[b,c], David Xi-an Li[d], Shaohui Zhang[a,b,*]

[a] School of Agriculture and Biology, Shanghai Jiao Tong University, China
[b] Zhejiang Go Peptides Life Science and Healthcare Technology Co., Ltd., China
[c] School of Systemic Biology and Medicine, Shanghai Jiao Tong University, China
[d] Zhejiang Panda Dairy Group Co. Ltd., China

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ABSTRACT

The present study here establishes a complete and effective method for isolating, purifying and identifying extracellular and intracellular peptides, and also describes the characters and bioactivities of peptides from fermented casein with *Lactobacillus helveticus*. Intracellular peptides are much larger in quantity and more complex in composition than extracellular peptides, between which the correlation reveals proteolytic and metabolic mechanisms.In addition, totally 241 different peptide sequences were identified by Nano LC–MS/MS from casein (212) and *Lactobacillus helveticus* proteins (29). These casein-derived peptides mostly originated from β-casein, followed by αS1-casein, κ-casein, and αS2-casein, and came from extracell (69) and intracell (143), in which common peptides have a total of 27. Forty-four of the identified peptides were previously described as bioactive, including angiotensin-converting enzyme (ACE)-inhibitory, antioxidant, immunomodulating, antimicrobial, DPP-IV inhibitory, antiamnesic and anticancer effects and so on. Thirteen peptides with the potential of some biological activities are obtained, which were described in previous studies. A total of 47 novel peptides of 5 to 26 amino acids that were not disclosed were obtained. The new sources of natural bioactive peptides may have the very high application value as potential new peptide drugs for treatment human diseases. The product peptide DELQDKIHPF found in both extracell and intracell was quantitatively analyzed using the MRM mode of UPLC-U3Q, 23.1 and 9.76 ng/mL, respectively. The quantitative analysis of the potential bioactive peptide may also advance the production of peptide products in the future.

1. Introduction

Protein is the material basis of life, and all life events need to have protein participation. Bioactive peptides have been defined as "specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health" [1]. A large number of bioactive peptides from animals or plants have been identified, which belong to current diet. For example, there were many bioactive peptides derived from food protein sources having been reported, such as from fermented milk [2], cheddar cheeses [3], hen egg white [4], tuna [5], soybean [6], rice [7], peanut [8], flaxseed [9], and pumpkin [10]. But milk proteins are still the major source of bioactive peptides and these peptides exhibit a wide range of biological activities [11]. Most of these bioactive peptides are derived from the degradation of casein. A number of studies showed that casein act as an excellent substrate to produce a variety of bioactive peptides with angiotensin I converting enzyme (ACE)-inhibitory, immunological, antioxidant, antithrombotic or opioid effects [12,13]. In a recent study, medium enriched with casein as a sole nitrogen source (comprised of 3% casein, 5%

* Corresponding author at: School of Agriculture and Biology, Shanghai Jiao Tong University, China.
E-mail address: 18817956370@163.com (S. Zhang).
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lactose and 1.17% yeast carbon base) was fermented for 42 h with each of two lactic acid bacteria isolated from the commercial fermented milk Soful (Yakult®, Mexico) to produce ACE-inhibitory peptides [14]. The use of casein or lactoferrin as a sole nitrogen source to dentify the ACE-inhibitory peptides released by yeasts has been recently described [15]. Compared to synthesed peptides, bioactive peptides produced by microbial fermentation are considered safer and healthier, without side effects, as they are derived from edible food proteins.

The approach to the production of these bioactive peptides involves the choice of microbial species. At present, the main strains are from Lactobacilli, Lactococcus, Leuconostoc and acetic acid bacteria, as well as different types of yeasts, and even kefir [16–18]. Many studies have focused on the potential of LAB to release bioactive peptides with milk proteins [19]. Compared to other lactic acid bacteria, *Lactobacillus helveticus* has a strong proteolytic activity with the most efficient proteolytic system, so it has been widely used in the food industry, especially in the dairy industry [20,21]. The bioactive peptides VPP and IPP from β-casein were isolated and identified from the fermented milk with *Lactobacillus helveticus CP790*, and the significant ACE-inhibitory effect *in vitro* was tested [22,23]. To achieve maximum production of bioactive peptides, it is necessary to find the optimal microbial species and fermentation conditions.

Many studies using lactic acid bacteria also have demonstrated their ability to produce different bioactive peptides during the fermentation process by their surface specific proteolytic systems, especially cell envelope proteinases (CEPs) [24]. The product is far more complex than imagined after different microbial hydrolysis. 236 peptides were uniquely detected in milk fermented with kefir, but not in raw milk, which indicated that the fermentation increase the proteolytic activity and change the complexity of composition of the peptide fraction [25]. H.E. Hatmi et al. [26] revealed the presence of 347 peptides in camel milk fermented with the proteolytic strain LMD-9 of *S. Thermophilus* and investigated their free radical scavenging power. Most of the peptides in their studies were derived from the four individual CNs, especially from β-CN, the major component. However, less is known about the composition and biological activity of total peptides, the relationship of extracellular and intracellular peptides and the connection between casein-derived peptides and bacterial protein-derived peptides.

In our previous work, we studied casein fermented by *Lactobacillus helveticus CICC6024* at optimal process conditions, followed by the isolation and identification of novel casein-derived bioactive peptides. In addition, the comprehensive analysis of the total casein-derived peptide profile in both extracell and intracell is carried out, including composition of the peptide fractions revealing information about the mechanisms of peptide formation. Finally, the potential bioactivities of novel casein-derived bioactive peptides and quantification of the target peptide sequence are discussed and implemented.

2. Materials and methods

2.1. Materials

Casein, lactose and Man, Rogosa and Sharpe (MRS) medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amicon Ultra-15 regenerated cellulose filters with a molecular weight (MW) cut-off of 3 kDa were obtained from Millipore (Carrigtwohill, Ireland). Purified target peptide DELQDKIHPF (above 95% purity) were synthesized by Shanghai Top-peptide Co., Ltd (Shanghai, China). All other chemicals were of the highest grade available commercially.

2.2. Microorganism and culture conditions

The *Lactobacillus helveticus* CICC6024 was placed in a 37 °incubator for 5 min and activated at 37 ° in MRS medium for 2–3 times. The medium of casein concentration 8%, lactose concentration 6% and NaCl 0.5% were prepared respectively, 65 ° water bath with 1 mol/L NaOH solution to adjust the pH to 6.5 to make casein dissolved, 115 ° high temperature sterilization for 15 min after 1 h water bath. Pre-cultures were prepared by inoculating the activated *Lactobacillus helveticus* CICC6024 with 6% of inoculum level in sterile casein mediun and incubated at 37°for 7 h to obtain a fermentation broth.

2.3. Preparation of peptide fractions from fermentation broth and Lactobacillus helveticus *cells*

After centrifugation at 4000 rpm/min for 15 min and 20°, the supernatant that is extracellular peptides extract was collected. At the same time, cells from fermented casein sample were collected and washed three times with 50 mmol/L Tris−HCl (pH7.1) by centrifugation at 4000↘4500↘5000rpm/min (20 °, 15 min) respectively. The washed cells were suspended in cell lysis buffer (Beyotime ®Biotechnology, China) and 100 mM PMSF (Beyotime ®Biotechnology, China), 0° incubating for 30 min and further rushed by ultrasonic treatment (200 W) at 3 s intervals for 10 min in an ice bath. The crude homogenate was centrifuged at 9000 rpm/min for 20 min at 4 °. The supernatant was used as a intracellular peptides extract. The supernatants of extracellular and intracellular extracts obtained were subjected to ultrafiltration centrifugation using centrifugal filter units with a 3 kDa cutoff (Carrigtwohill, Ireland) at 4800 r/min for 30 min and 4°for the removal of macromolecule casein.

Before solid-phase extraction (SPE), the ultrafiltrate was diluted 10 times with ddH_2O. Solid phase extraction conditions were modificated slightly according to C. ClarianaS [27] by means of C18-SPE (100 mg C18-E, 1 mL,Waters, USA). SPE columns were first conditioned with 2 mL of methanol and 2 mL of water. After loading 2 mL of ultrafiltrate samples, the retained compounds were eluted with 400 μL of eluent (80:20 v/v methanol/water and 0.1% v/v formic acid). During all the above steps, control the flow rate of approximately 1 mL/min. The eluent was evaporated with nitrogen blowing instrument at room temperature to about 1–2 mL for concentration, and then it was loaded into EP tubes and vacuum frozen for 12 h and dried to a powder and stored at -80° for spare.

2.4. Gastrointestinal digestion in vitro

The samples were lyophilized into powders followed by gastrointestinal digestion simulation whose main steps referred to R. JÁG et al. [28] and were modified according to M.M. Contreras et al. [29]. Specific Methods: 1.5 mg of sample powders was dissolved in 1.5 mL of ddH_2O. Pepsin solution was added into sample solution (enzyme: substrate = 1:50, w/w), and the pH was adjusted to 2.0, water bathing at 37°for 90 min. Subsequently, the pH was adjusted to 7.5 and trypsin solution was added (enzyme: substrate = 1: 25, w/w), water bathing at 37°for 150 min. Finally, the enzyme was inactivate in 95°hot water bathing for 5 min to make enzyme reaction terminated. The solution was freezing-dried into powders, stored at -80° for subsequent analysis.

2.5. Total peptides quantification

The total peptides concentration was measured by Lowry [30] assay with peptone [31] as a standard (Lowry Protein Assay Kit, Shanghai Sangon Biotech).

2.6. Identification of peptides by Nano LC–MS/MS

The peptide samples was analyzed using an LC system (Nano Pump, Ultimate 3000, Dionex, Thermofisher) coupled with an ESI-Q-TOFmass spectrometer (maXis, Impact, Bruker Daltonik, Germany). To desalt and concentrate samples, each peptide sample was re-dissolved in UP H_2O with 0.1% formic acid and shaken for 5–10 min, followed by high-speed centrifugation at 12,000 r/min for 10 min. The Millipore C18 ZipTip was equilibrated with 0.1% FA and ACN, followed by loading peptide samples. Then the samples were washed with 0.1% FA and eluted with 20% ACN, 0.1% FA. The eluted sample was dried and further dissolved with 2% ACN, 0.1% FA and analyzed by NanoLC-MS with C18 capillary column (75 μm × 15 cm, 3 μm, Dionex, Thermofisher). The elution system consisted of A: 0.1% (v/v) formic acid (TFA) in water and B: 0.1% (v/v) formic acid in acetonitrile. 1 μL sample was injected and eluted at a flow rate of 0.25 μL/min with a linear gradient as follows:0–10 min, 2% B; 10–12 min, 4%–8% B; 12–110min, 8%–20% B; 110–125 min, 20%–30% B; 125–125.1min, 30%–80% B; 125.1–135 min, 80% B; 135–135.1min, 80%–2% B; 135.1–150 min, 2% B. The LC setup was coupled online to a Q-TOF using a nano-ESI source (Bruker Daltonik, Germany) in data dependent acquisition (DDA) mode (*m/z* 100–1500). The Source Capillary was set at 2000 v, the flow and temperature of dry gas was 2.0 L/min and 150 °C respectively. The mass spectrometerwas set as one fullMSscan followed by tenMS/MS scans on the tenmost intense ions from the MS spectrum. Peptide charges of +1, +2, and +3 were selected in each scan.The results from the product ion scans were subjected to a MASCOT (version 2.4, Matrix Science) search against database for peptide identification.

2.7. Database search and peptide profile analysis

Mascot (version 2.4, Matrix Science) was set up to search casein database and *Lactobacillus helveticus CICC6024* protein database which was established according to genome sequence. The database of casein amino acid sequence was established by searching BIOPEP [32] and *Structure-function properties of food proteins* [33], including α_{s1}- casein (ID: 1086–1089, variant A–D), α_{s2}-casein (ID:1090, variants A1), β- casein (ID:1097-1103, variant A1-A3, B, C, E, F), κ- casein (ID:1117, variant A). The amino acid sequences obtained were sorted out and compiled into the first database, generating a fasta file. The amino acid sequence of *Lactobacillus helveticus CICC6024* protein was obtained by RNA gene sequencing and genome sequencing, then translating into amino acid sequence of possibly expressed proteins. The second database was constructed with generating the second fasta file.

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Compass Data Analysis version 4.1 (Bruker Daltonics), generating mgf files. The Mascot 2.4 search engine was set up to search for the above two protein amino acid databases. Search parameters were: Carbamidomethyl (C) as fixed modifications, Oxidation (M) as variable modifications, no enzyme, two missed cleavages, 20 ppm mass tolerance for precursor ions and 0.5 Da for product ions, charge minimum +1. For reliable peptide identification, the following approach was applied: Significance threshold was set as 0.05 and all peptides with a score >15 were preselected. At least one unique peptide can be detected from one protein.

2.8. Selection and absolute quantification of target peptide

2.8.1. Target peptide

According to the analysis results above, select the common peptide DELQDKIHPF f(43–52) from β-casein var B among the extracellular and intracellular peptides, whose dupes was relatively high in mass spectrometry results that means its content of the total peptides was same relatively high. Therefore, the synthesized peptide DELQDKIHPF was synthesized as a standard peptide to quantify the same peptide in the sample by the synthesizer Shanghai Top-peptide Co., Ltd (Shanghai, China).

Table 1
MRM transitions for DELQDKIHPF.

Sequence	Precursor ion (m/z)	Product ion (m/z)	Fragment
DELQDKIHPF	621.500	263.200 245.000	y2 b2

2.8.2. Peptide absolute quantification by UPLC-3Q-MS/MS

The separation of peptides was carried out using the ACQUITY UPLC System equipped with ACQUITY UPLC Binary Solvent Manager, ACQUITY UPLC Sample Manager, and ACQUITY UPLC Column Manager (Waters, Milford, MA, USA). The Column was ACQUITY UPLC BEH300 C18 column (1.7 μm particle size, 2.1 × 100 mm) reversed phase analytical column (Waters, Milford, MA, USA), and equipped with a guard column of the same material. Mobile phase A was 100% H_2O and mobile phase B was 100% acetonitrile. The injection volume was 5 μL. The flow rate of the mobile phase was 0.4 mL/min. The column temperature was 35 °C. The LC elution program was linear gradient as follows: 0–0.5 min, 18% B; 0.5–3.5 min, 18% B; 3.5–4 min, 85%B; 4 −6 min, 100% B; 6–6.5 min, 100% B; 6.5–8 min, 18% B. The total injection cycling time was 8 min. Ion Source (Turbo Spray), Curtain Gas (35 Psi), Collision Gas (8 Psi), IonSpay Voltage (5500 V), Desolvation Temperature (500°), Ion Source Gas1 (50 Psi) and Ion Source Gas2 (50 Psi) were optimized for MRM intensity derived from DELQDKIHPF. Precursor/product ion and their corresponding fragment for target peptide was created in the MRM transition list (Table 1).

Six-point calibration curve of the pure peptide DELQDKIHPF was constructed as follows. The synthetic peptide DELQDKIHPF were solubilized in ultrapure water, and then diluted with UP H_2O, to obtain the 2, 5, 10, 15, 20 and 25 ng/mL solutions. The calibration curve was constructed from the peak area of the standard peptide versus concentration. The concentrations of DELQDKIHPF in two samples were calculated using a linear equation.

3. Results and Discussion

3.1. Peptide analysis by Nano LC–MS/MS

A detailed peptide analysis was obtained by direct Nano LC–MS/MS measurement in the *m/z* range from 100 to 1500 Da. Fig. 1 shows specific Nano LC–MS/MS spectra for extracellular peptides and intracellular peptides. From the comparison with Fig. 1**A** and **B**, we can see the peaks of Fig. 1 **B** are larger in amount than Fig. 1**A**. It means that intracellular peptides are much more complicated than extracellular peptides. Intracell is superior to extracell on the number of peptides producted by *Lactobacillus helveticus*. This result is strongly related to the type and amount of intracellular peptidases. The proteolytic system of lactic acid bacteria consists of three parts: cell wall proteases, different types of amino acid and oligopeptide transport systems, as well as a pool of intracellular peptidases (endopeptidase, aminopeptidase, proline-specific enzyme, tripeptidase and dipeptidases). To make use of casein in milk, lactic acid bacteria firstly hydrolyze casein into oligopeptides by cell wall proteases and then transport it into cells through a specific oligopeptide transport system, further degrading oligopeptides into smaller peptides and amino acids by intracellular peptidases to provide for the growth and utilization of bacteria [34,35]. At the same time, intracellular peptides are not only from casein hydrolysis, but also from the *Lactobacillus helveticus* itself

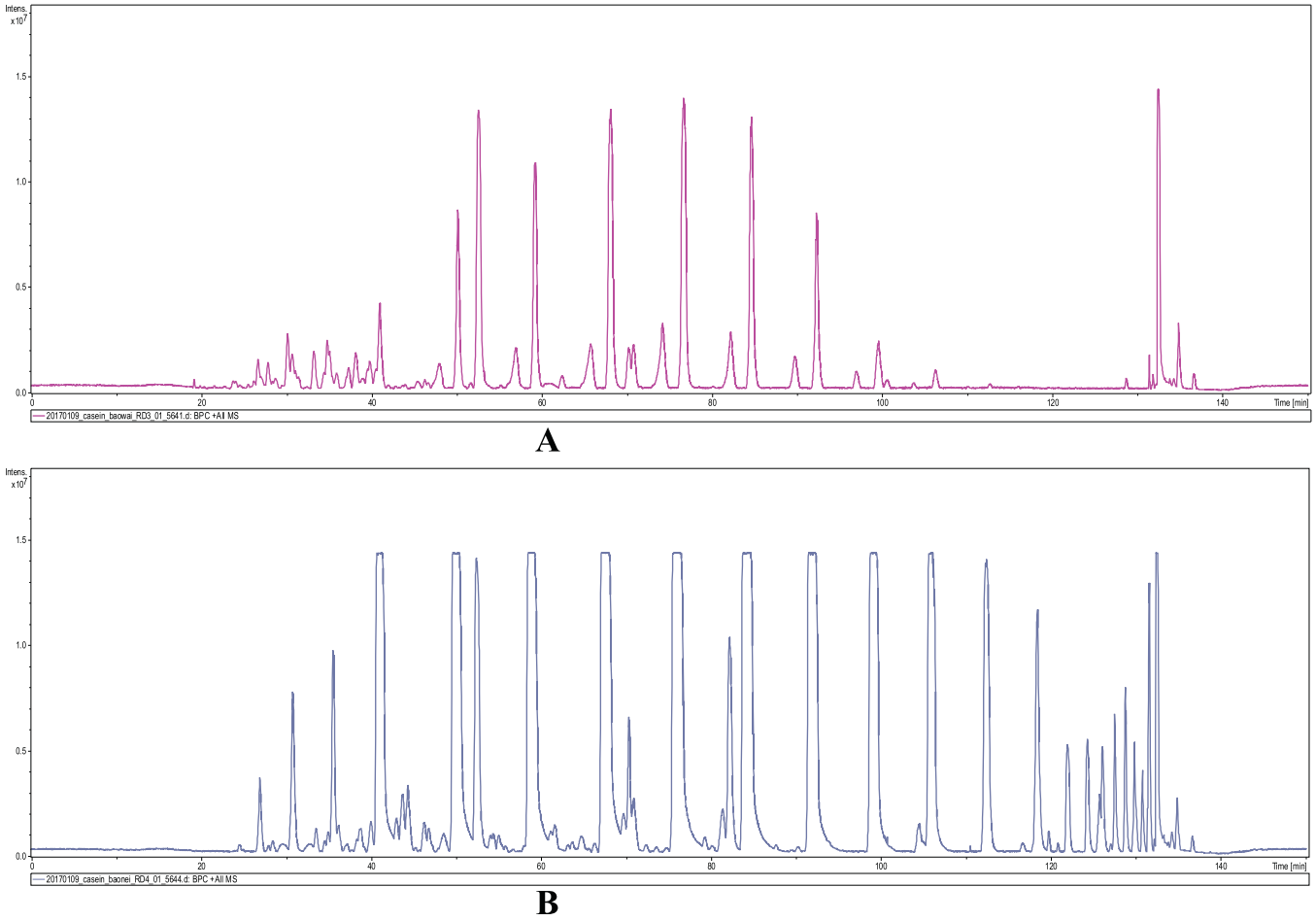


Fig. 1. Nano LC–MS/MS mass spectra of extracellular peptides (A) and intracellular peptides (B) fermented with *Lactobacillus helveticus* in a mass range from *m/z* 100 to 1500.

because of existing bacteria protein degradation. The peptide profiles recorded by Nano LC give a good overview of predominant peptides.

To identificate specific peptides, a MASCOT database search was applied for interpretation of the fragment spectra. All resulting assigned peptide sequences of casein and bacterial proteins were manually checked the p value less than 0.05 to achieve unambiguous identification. As a result, totally 241 different peptide sequences were confirmed from extracell and intracell as Table 2. Including peptide sequence, precursor protein, position, and origin. The sequences of the identified peptides ranged from five to twenty-six amino acids.

Our work eventually obtained all bioactive peptides by fermenting casein using *Lactobacillus helveticus*, which resulted in a specific ṗeptide databaseẗhat contained 241 peptide sequences derived from variants of casein and *Lactobacillus helveticus* proteins to be established, the casein-derived peptides including those derived from extracell and intracell of *Lactobacillus helveticus*. We published the ṗeptide databaseẗlike a large v̈ault;̈ not only including a variety of peptide fragments with biological activities, but containing proteins metabolism mechanism and potential research value during the evolution of human life. Bioactive peptides beneficial to the high-grade animals found from the lower organisms at bacterial level are whether to be reserved by n̈atural selection, survival of the fittestïn the process of biological evolution from low-grade bacteria to high-grade animals, which play an important functional role in its life-sustaining physiological activities. Whether the original genetic information of some proteins with special functions is preserved in the process of biological evolution or not contains deep scientific significance, worthy of further exploration and research.

3.2. Peptide profile analysis

Fig. 2**A** shows total 241 different peptide sequences were found, among which 212 peptides were from casein and 29 peptides came from *Lactobacillus helveticus* protein. These casein-derived peptides originated from β-casein (105), followed by α_{S1}-casein (60), κ-casein (26), and α_{S2}-casein (21). And the proportion was 49.5% for β-casein, 28.3% for α_{S1}-casein, 12.3% for κ-casein, and about 9.9% for α_{S2}-casein, which shows that peptides from β-casein accounted for the majority. However, in milk proteins 34% of casein is α_{S1}-casein, followed by 25% β-casein, 9% κ-caseins, and 8% α_{S2}-caseins [103]. Therefore, the number of released peptides was not related to the abundance of the parent proteins but to the preferences of the proteases. β-casein must be prone to enzymatic degradation by *Lactobacillus helveticus* and release more active peptides that benefit to microbial survival. The hydrolysis of α_{S1}-, α_{S2}- and β-casein by highly proteolytic strains *S. Thermophilus* to release bioactive peptides was studied, generating the maximum number of peptides from β-casein, secondly α_{S2}-casein and lastly α_{S1}-casein [104].

Among these casein-derived biological active peptides, intracellular peptides were much more complex than extracellular peptides in terms of whether type or quantity considerations, and 69 extracellular peptides and 143 intracellular peptides were identified. The number of the intracellular peptides was more 2 - fold more than that of extracellular peptides, which is related to microbial unique proteolytic system. In addition, the average number of

Table 2
Intracellular and extracellular identified sequences in experimental groups.

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
1	707.8632	1413.7118	2	α S1-casein var C	182-195	IPNPIGSENSGKTT	●				
2	886.4525	1770.8904	2	α S1-casein var C	182-198	IPNPIGSENSGKTTMPL	●				
3	716.3632	1430.7118	2	α S1-casein var C	185-198	PIGSENSGKTTMPL	●				
4	715.8454	1429.6762	2	α S1-casein var B	176-189	APSFSDIPNPIGSE	●		√	–	[35]
5	792.9072	1583.7999	2	α S1-casein var B	129-142	AQQKEPMIGVNQEL	●		√	–	[35]
6	828.4299	1654.8452	2	α S1-casein var B	129-143	AQQKEPMIGVNQELA	●				
7	941.457	940.4497	1	α S1-casein var B	181-189	DIPNPIGSE	●				
8	604.7715	1207.5284	2	α S1-casein var D	66-75	DQAMEDIKQM	●		√	–	[36]
9	489.7525	977.4905	2	α S1-casein var B	85-92	DVPSERYL	●		√	–	[37]
10	518.2624	1034.5102	2	α S1-casein var B	85-93	DVPSERYLG	●		√	ACE-inhibitory	[38]
11	943.5096	942.5024	1	α S1-casein var B	14-21	EVLNENLL	●	●	√	–	[35]
12	550.3106	1098.6067	2	α S1-casein var B	14-22	EVLNENLLR	●	●	√	–	[35]
13	806.4079	805.4006	1	α S1-casein var D	39-45	FVAPFPE	●	●	√	ACE-inhibitory	[39]
14	905.4759	904.4686	1	α S1-casein var D	39-46	FVAPFPEV	●		√	ACE-inhibitory	[40]
15	826.4295	825.4222	1	α S1-casein var B	182-189	IPNPIGSE	●		√	–	[35]
16	940.4735	939.4662	1	α S1-casein var B	182-190	IPNPIGSEN	●		√	–	[36]
17	578.7781	1155.5417	2	α S1-casein var B	182-192	IPNPIGSENSE	●		√	–	[35]
18	693.3494	1384.6842	2	α S1-casein var B	182-194	IPNPIGSENSEKT	●		√	–	[41]
19	914.4608	1826.907	2	α S1-casein var B	182-198	IPNPIGSENSEKTTMPL	●		√	–	[42]
20	664.8516	1327.6887	2	α S1-casein var B	132-143	KEPMIGVNQELA	●				
21	809.3987	1616.7829	2	α S1-casein var B	184-198	NPIGSENSEKTTMPL	●				
22	752.3774	1502.7403	2	α S1-casein var B	185-198	PIGSENSEKTTMPL	●				
23	853.4102	1704.8059	2	α S1-casein var B	185-199	PIGSENSEKTTMPLW	●	●	√	potential anti-hypertensive	[43]
24	1159.5321	1158.5248	1	α S1-casein var B	140-148	QELAYFYPE	●		√	–	[100]
25	693.3623	1384.7101	2	α S1-casein var B	131-142	QKEPMIGVNQEL	●		√	–	[35]
26	736.877	1471.7394	2	α S1-casein var B	131-143	QKEPMIGVNQELA	●	●	√	–	[44]

27	812.4512	811.4439	1	α S1-casein var B	108-114	QLEIVPN	●	●	√	–	[45]
28	755.393	754.3857	1	α S1-casein var B	180-186	SDIPNPI	●		√	–	[46]
29	1028.4877	1027.4804	1	α S1-casein var B	180-189	SDIPNPIGSE	●		√	–	[35]
30	679.8082	1357.6019	2	α S1-casein var B	180-192	SDIPNPIGSENSE	●		√	–	[47]
31	631.8022	1261.5898	2	α S1-casein var B	178-189	SFSDIPNPIGSE	●				
32	796.8597	1591.7048	2	α S1-casein var B	178-192	SFSDIPNPIGSENSE	●	●	√	–	[35]
33	758.4081	757.4008	1	α S1-casein var D	40-46	VAPFPEV	●	●	√	potential DPP IV inhibitor	[48]
34	610.3204	1218.6262	2	α S1-casein var D	40-50	VAPFPEVFGKE	●	●	√	ACE-inhibitory	[40]
35	496.269	990.5235	2	α S1-casein var D	41-49	APFPEVFGK		●	√	–	[36]
36	560.7897	1119.5648	2	α S1-casein var D	41-50	APFPEVFGKE		●	√	–	[36]
37	554.2722	1106.5299	2	α S1-casein var B	84-92	EDVPSERYL		●	√	–	[37]
38	476.7432	951.4719	2	α S1-casein var D	43-50	FPEVFGKE		●	√	–	[49]
39	753.3468	1504.6791	2	α S1-casein var B	179-192	FSDIPNPIGSENSE		●	√	–	[35]
40	569.979	1706.9152	3	α S1-casein var D	39-53	FVAPFPEVFGKEKVN		●	√	–	[35]
41	567.8017	1133.5888	2	α S1-casein var B	8-17	HQGLPQEVLN		●	√	–	[50]
42	509.7905	1017.5665	2	α S1-casein var B	16-23	LNENLLRF		●	√	immunomodulating;antibacterial;anticancer	[51,52,53]
43	453.2456	904.4766	2	α S1-casein var B	17-23	NENLLRF		●	√	–	[54]
44	997.5331	996.5258	1	α S1-casein var B	9-17	QGLPQEVLN		●	√	–	[46]
45	810.4184	1618.8222	2	α S1-casein var B	131-144	QKEPMIGVNQELAY		●	√	–	[35]
46	659.3408	658.3335	1	α S1-casein var D	40-45	VAPFPE		●	√	potential DPP IV inhibitor	[55]
47	545.8013	1089.5881	2	α S1-casein var D	40-49	VAPFPEVFGK		●	√	potential DPP IV inhibitor	[55]
48	780.926	1559.8375	2	α S1-casein var D	40-53	VAPFPEVFGKEKVN		●	√	–	[50]
49	485.7896	969.5647	2	α S1-casein var B	15-22	VLNENLLR		●	√	antibacterial	[56]
50	559.3231	1116.6316	2	α S1-casein var B	15-23	VLNENLLRF		●	√	–	[35]
51	1008.574	1007.5667	1	α S1-casein var B	106-114	VPQLEIVPN		●	√	potential DPP IV inhibitor	[55]

Table 2 (*Continued*)

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
52	811.4951	810.4878	1	α S2-casein var A	131-138	AVPITPTL	●	●	√	–	[44]
53	925.5407	924.5334	1	α S2-casein var A	131-139	AVPITPTLN	●	●	√	–	[44]
54	605.8468	1209.6791	2	α S2-casein var A	131-141	AVPITPTLNRE	●	●	√	–	[36]
55	669.8736	1337.7326	2	α S2-casein var A	131-142	AVPITPTLNREQ	●	●			
56	925.5369	924.5296	1	α S2-casein var A	130-138	NAVPITPTL		●	√	antioxidative	[57]
57	528.2476	1054.4807	2	α S2-casein var A	98-105	NEINQFYQ		●			
58	778.8757	1555.7368	2	α S2-casein var A	98-109	NEINQFYQKFPQ		●			
59	747.4118	1492.8091	2	α S2-casein var A	116-128	QGPIVLNPWDQVK		●	√	–	[35]
60	550.6467	1648.9182	3	α S2-casein var A	116-129	QGPIVLNPWDQVKR		●	√	–	[35]
61	588.6613	1762.9619	3	α S2-casein var A	116-130	QGPIVLNPWDQVKRN		●	√	–	[44]
62	602.3307	1202.6469	2	α S2-casein var A	79-89	VATEEVKITVD		●			
63	740.4572	739.4499	1	α S2-casein var A	132-138	VPITPTL		●	√	DPP IV inhibitor	[58]
64	854.4977	853.4904	1	α S2-casein var A	132-139	VPITPTLN		●			
65	505.8033	1009.5921	2	α S2-casein var A	132-140	VPITPTLNR		●			
66	570.3278	1138.641	2	α S2-casein var A	132-141	VPITPTLNRE		●			
67	634.3581	1266.7016	2	α S2-casein var A	132-142	VPITPTLNREQ		●	√	–	[59]
68	643.014	1926.0201	3	α S2-casein var A	115-130	YQGPIVLNPWDQVKRN		●	√	–	[50]
69	974.5567	973.5494	1	β-casein var B	191-198	LLYQEPVL	●		√	potential ACE-inhibitory	[44]
70	861.4722	860.465	1	β-casein var B	192-198	LYQEPVL	●	●	√	improve the growth of human keratinocytes in culture	[60]
71	488.7625	975.5105	2	β-casein var B	112-119	PKYPVEPF	●				
72	748.3878	747.3805	1	β-casein var B	193-198	YQEPVL	●	●	√	ACE inhibitor	[61]
73	621.3135	1240.6124	2	β-casein var B	43-52	DELQDKIHPF	●	●	√	potential Anti-hypertensive	[62]
74	563.8017	1125.5888	2	β-casein var B	44-52	ELQDKIHPF	●	●	√	Antimicrobial	[63]
75	588.3773	587.37	1	β-casein var B	205-209	FPIIV	●		√	ACE inhibitor	[64]

76	787.4357	786.4285	1	β-casein var B	157-163	FPPQSVL	●		√	ACE-inhibitory	[40]
77	470.2744	938.5343	2	β-casein var B	199-207	GPVRGPFPI	●	●	√	immunomodulating;ACE-inhibitor;antioxidative	[65,66,67]
78	576.3531	1150.6916	2	β-casein var B	199-209	GPVRGPFPIIV	●		√	ACE-inhibitory	[40]
79	883.4833	882.476	1	β-casein var B	73-80	NIPPLTQT	●		√	potential antioxidant	[68]
80	619.2988	1854.8745	3	β-casein var B	38-52	QQQTEDELQDKIHPF	●	●			
81	1005.5126	1004.5053	1	β-casein var B	123-131	QSLTLTDVE	●	●			
82	578.3062	1154.5978	2	β-casein var B	1-10	RELEELNVPG	●		√	–	[36]
83	642.829	1283.6434	2	β-casein var B	1-11	RELEELNVPGE	●		√	–	[36]
84	877.4526	876.4453	1	β-casein var B	124-131	SLTLTDVE	●	●	√	–	[35]
85	991.4934	990.4861	1	β-casein var B	124-132	SLTLTDVEN	●	●	√	–	[35]
86	879.4581	878.4508	1	β-casein var B	57-64	SLVYPFPG	●		√	–	[69]
87	670.8578	1339.701	2	β-casein var A1	57-68	SLVYPFPGPIHN	●	●	√	ACE-inhibitory	[70]
88	1092.5382	1091.5309	1	β-casein var A1	122-131	SQSLTLTDVE	●	●	√	–	[71]
89	661.8218	1321.629	2	β-casein var A1	120-131	TESQSLTLTDVE	●		√	–	[36]
90	558.3276	557.3204	1	β-casein var B	83-87	VVPPF	●		√	potential ACE-inhibitory	[72]
91	671.4128	670.4056	1	β-casein var B	83-88	VVPPFL	●				
92	1025.5654	1024.5582	1	β-casein var B	83-91	VVPPFLQPE	●		√	–	[49]
93	570.7963	1139.578	2	β-casein var A1	59-68	VYPFPGPIHN	●		√	ACE-inhibitory;antioxidative	[73]
94	884.959	1767.9034	2	β-casein var A1	53-68	AQTQSLVYPFPGPIHN		●			
95	552.3776	551.3703	1	β-casein var A1	135-139	LPPLL		●			
96	627.3399	1252.6653	2	β-casein var A1	58-68	LVYPFPGPIHN		●	√	Bitter Peptides	[74]
97	998.5093	2992.5062	3	β-casein var A3	89-114	QPEVMGVSKV-KEAMAPKQKEMPFPKY		●			
98	510.7985	1019.5824	2	β-casein var A1	195-204	QPVLGPVRGP		●			
99	632.8582	1263.7019	2	β-casein var A1	195-206	QPVLGPVRGPFP		●	√	–	[45]
100	574.8243	1147.6341	2	β-casein var A1	194-204	QQPVLGPVRGP		●			
101	542.3077	1082.6008	2	β-casein var A1	166-175	SQSKVLPVPE		●			
102	521.2649	1040.5152	2	β-casein var A1	60-68	YPFPGPIHN		●	√	ACE-inhibitory	[75]
103	564.7807	1127.5469	2	β-casein var A1	60-69	YPFPGPIHNS		●	√	Bitter Peptides	[74]
104	733.8768	1465.7391	2	β-casein var A1	60-72	YPFPGPIHNSLPQ		●	√	Opioid agonist	[76]
105	557.2813	1668.8221	3	β-casein var B	43-56	DELQDKIHPFAQTQ		●	√	potential Antihypertensive	[62]
106	837.3801	836.3728	1	β-casein var B	184-190	DMPIQAF		●	√	–	[35]
107	685.8353	1369.656	2	β-casein var B	42-52	EDELQDKIHPF		●	√	–	[36]
108	886.4207	885.4134	1	β-casein var B	4-11	EELNVPGE		●	√	–	[49]
109	777.9039	1553.7932	2	β-casein var B	44-56	ELQDKIHPFAQTQ		●	√	–	[36]
110	510.7985	1019.5824	2	β-casein var B	195-204	EPVLGPVRGP		●	√	cytomodulatory effect	[77]
111	632.8582	1263.7019	2	β-casein var B	195-206	EPVLGPVRGPFP		●	√	immunomodulating	[78]
112	741.3911	740.3838	1	β-casein var B	62-68	FPGPIPN		●	√	DPP IV inhibitor	[79]
113	828.4213	827.414	1	β-casein var B	62-69	FPGPIPNS		●			
114	563.288	1124.5615	2	β-casein var B	111-119	FPKYPVEPF		●			
115	677.3436	1352.6727	2	β-casein var B	111-121	FPKYPVEPFTE		●	√	–	[80]

Table 2 (*Continued*)

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
116	874.4712	873.4639	1	β-casein var B	157-164	FPPQSVLS		●			
117	689.4349	688.4276	1	β-casein var B	134-139	HLPLPL		●	√	antiamnesic	[81]
118	965.5669	964.5596	1	β-casein var B	74-82	IPPLTQTPV		●	√	DPP IV inhibitor	[79]
119	670.4135	669.4062	1	β-casein var B	137-142	LPLLQS		●	√	–	[82]
120	668.8853	1335.7561	2	β-casein var B	171-182	LPVPQKAVPYPQ		●	√	–	[50]
121	598.3593	1194.7041	2	β-casein var B	165-175	LSQSKVLPVPQ		●	√	ACE-inhibitory	[83]
122	904.4598	903.4526	1	β-casein var B	125-132	LTLTDVEN		●	√	–	[82]
123	722.3572	721.35	1	β-casein var B	185-190	MPIQAF		●	√	antibacterial	[84]
124	1079.6117	1078.6044	1	β-casein var B	73-82	NIPPLTQTPV		●	√	ACE inhibitor	[85]
125	643.3336	642.3263	1	β-casein var B	9-14	PGEIVE		●	√	–	[49]
126	603.8073	1205.6	2	β-casein var B	112-121	PKYPVEPFTE		●	√	inhibition of 15-lipoxygenase	[86]
127	552.3776	551.3703	1	β-casein var B	136-140	PLPLL		●	√	ACE inhibitor	[87]
128	827.4266	826.4193	1	β-casein var B	85-91	PPFLQPE		●	√	–	[46]
129	601.8063	1201.598	2	β-casein var B	181-190	PQRDMPIQAF		●	√	–	[35]
130	547.843	1093.6714	2	β-casein var B	200-209	PVRGPFPIIV		●	√	Bitter Peptides	[88]
131	838.468	837.4607	1	β-casein var B	194-201	QEPVLGPV		●	√	–	[49]
132	574.8243	1147.6341	2	β-casein var B	194-204	QEPVLGPVRGP		●	√	–	[50]
133	696.8898	1391.7651	2	β-casein var B	194-206	QEPVLGPVRGPFP		●	√	immunomodulating;ACE-inhibitor;antioxidative	[78]
134	630.8249	1259.6352	2	β-casein var B	146-156	QPHQPLPPTVM		●	√	–	[101]
135	564.2966	1126.5786	2	β-casein var B	149-158	QPLPPTVMFP		●	√	inhibitors of apoptosis and other cellular damage	[89]
136	533.931	1598.7711	3	β-casein var B	40-52	QTEDELQDKIHPF		●	√	–	[58]
137	813.429	1624.8435	2	β-casein var B	1-14	RELEELNVPGEIVE		●	√	potential ACE-inhibitory	[43]
138	550.9955	1649.9646	3	β-casein var B	168-182	SKVLPVPQKAVPYPQ		●	√	potential antioxidant	[62]
139	641.8748	1281.735	2	β-casein var B	164-175	SLSQSKVLPVPQ		●	√	–	[49]
140	689.3974	2065.1704	3	β-casein var B	164-182	SLSQSKVLPVPQKAVPYPQ		●	√	–	[35]
141	542.3077	1082.6008	2	β-casein var B	166-175	SQSKVLPVPQ		●	√	ACE inhibitors	[90]
142	933.5298	1865.045	2	β-casein var B	166-182	SQSKVLPVPQKAVPYPQ		●	√	–	[35]
143	491.2418	1470.7035	3	β-casein var B	41-52	TEDELQDKIHPF		●	√	–	[50]
144	696.3566	1390.6986	2	β-casein var B	120-131	TERQSLTLTDVE		●			
145	904.4724	903.4651	1	β-casein var B	126-133	TLTDVENL		●	√	–	[49]
146	819.4111	818.4039	1	β-casein var B	154-160	TVMFPPQ		●			
147	718.4198	1434.825	2	β-casein var B	170-182	VLPVPQKAVPYPQ		●	√	–	[35]
148	734.3551	733.3478	1	β-casein var B	155-160	VMFPPQ		●			
149	821.39	820.3827	1	β-casein var B	155-161	VMFPPQS		●	√	–	[82]
150	572.3452	571.3379	1	β-casein var B	84-88	VPPFL		●	√	potential antihypertensive;Bitter Peptides	[62,91]
151	926.499	925.4917	1	β-casein var B	84-91	VPPFLQPE		●	√	–	[35]

152	551.7747	1101.5349	2	β-casein var B	178-186	VPYPQRDMP		●	√	–	[42]
153	608.3177	1214.6209	2	β-casein var B	178-187	VPYPQRDMPI		●			
154	672.343	1342.6714	2	β-casein var B	178-188	VPYPQRDMPIQ		●	√	leptin-associated disorders	[92]
155	707.8652	1413.7159	2	β-casein var B	178-189	VPYPQRDMPIQA		●	√	ACE-inhibitory	[93]
156	781.4061	1560.7977	2	β-casein var B	178-190	VPYPQRDMPIQAF		●	√	–	[50]
157	1001.5115	1000.5042	1	β-casein var B	60-68	YPFPGPIPN		●	√	ACE inhibitor	[94]
158	683.3391	1364.6635	2	β-casein var B	180-190	YPQRDMPIQAF		●			
159	981.455	980.4477	1	β-casein var B	114-121	YPVEPFTE		●	√	ACE inhibitor	[95]
160	902.4626	901.4554	1	β-casein var B	193-200	YQEPVLGP		●	√	antioxidative	[96]
161	579.3216	1156.6286	2	β-casein var B	193-202	YQEPVLGPVR		●	√	ACE inhibitor	[97]
162	778.4208	1554.8271	2	β-casein var B	193-206	YQEPVLGPVRGPFP		●	√	immunomodulating	[78]
164	663.8472	1325.6799	2	κ-casein var A	172-183	EVIESPPEINTV	●				
165	612.3138	1222.6131	2	κ-casein var A	171-181	PEVIESPPEIN	●				
166	712.3719	1422.7292	2	κ-casein var A	171-183	PEVIESPPEINTV	●				
167	696.3331	1390.6516	2	κ-casein var A	145-158	TIASGEPTSTPTTE	●	●	√	–	[36]
168	997.5182	996.511	1	κ-casein var A	173-181	VIESPPEIN	●	●	√	–	[36]
169	477.7909	953.5672	2	κ-casein var A	87-95	AVRSPAQIL		●	√	antibacterial	[98]
170	541.8232	1081.6318	2	κ-casein var A	87-96	AVRSPAQILQ		●	√	–	[36]
171	614.3037	1226.5929	2	κ-casein var A	35-44	DERFFSDKIA		●			
172	1030.5479	1029.5406	1	κ-casein var A	136-144	DKTEIPTIN		●	√	–	[36]
173	563.7917	1125.5688	2	κ-casein var A	172-181	EVIESPPEIN	●	●	√	–	[99]
174	641.3308	1280.6471	2	κ-casein var A	60-70	GLNYYQQKPVA		●			
175	697.8743	1393.7341	2	κ-casein var A	60-71	GLNYYQQKPVAL		●			
176	898.4526	897.4453	1	κ-casein var A	174-181	IESPPEIN		●	√	–	[36]
177	899.9621	1797.9096	2	κ-casein var A	72-86	INNQFLPYPYYAKPA		●			
178	886.4363	885.4291	1	κ-casein var A	77-83	LPYPYYA		●			
179	591.8135	1181.6124	2	κ-casein var A	77-86	LPYPYYAKPA		●	√	–	[36]
180	843.4302	1684.8458	2	κ-casein var A	73-86	NNQFLPYPYYAKPA		●			
181	786.4012	1570.7878	2	κ-casein var A	74-86	NQFLPYPYYAKPA		●			
182	729.3805	1456.7464	2	κ-casein var A	75-86	QFLPYPYYAKPA		●	√	–	[36]
183	928.5105	927.5032	1	κ-casein var A	98-106	QVLSNTVPA		●			
184	655.834	1309.6534	2	κ-casein var A	170-181	SPEVIESPPEIN		●			
185	592.8174	1183.6202	2	κ-casein var A	176-186	SPPEINTVQVT		●			
186	722.862	1443.7094	2	κ-casein var A	59-70	YGLNYYQQKPVA		●	√	improve the growth of human keratinocytes in culture	[60]
187	978.5601	977.5528	1	>LBH_0027|m.24 LBH_0027|g.24 ORF LBH_0027|g.24 LBH_0027|m.24 type:5prime_partial len:320 (+) LBH_0027:1-960(+)		ATAVPIIFF		●			

Table 2 (*Continued*)

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
188	856.4322	855.4249	1	>LBH_0034|m.31 LBH_0034|g.31 ORF LBH_0034|g.31 LBH_0034|m.31 type:complete len:745 (+) LBH_0034:1-2235(+)		EPGIVNLD		●			
189	696.895	1391.7755	2	>LBH_0086|m.80 LBH_0086|g.80 ORF LBH_0086|g.80 LBH_0086|m.80 type:complete len:369 (+) LBH_0086:1-1107(+)		MQFAGLIILTIVG		●			
190	734.3551	733.3478	1	>LBH_0140|m.131 LBH_0140|g.131 ORF LBH_0140|g.131 LBH_0140|m.131 type:complete len:238 (+) LBH_0140:1-714(+)		GGEPFLN		●			
191	404.7307	807.4468	2	>LBH_0163|m.151 LBH_0163|g.151 ORF LBH_0163|g.151 LBH_0163|m.151 type:complete len:997 (+) LBH_0163:1-2991(+)		KVTPYQA		●			
192	722.86	1443.7055	2	>LBH_0185|m.172 LBH_0185|g.172 ORF LBH_0185|g.172 LBH_0185|m.172 type:complete len:462 (+) LBH_0185:1-1386(+)		ADVKIGNDTVIEGN		●			

193	863.4365	862.4292	1	>LBH_0232|m.214 LBH_0232|g.214 ORF LBH_0232|g.214 LBH_0232|m.214 type:5prime_partial len:343 (+) LBH_0232:1-1029(+)	IYQMVHA	●
194	775.4212	774.4139	1	>LBH_0240|m.224 LBH_0240|g.224 ORF LBH_0240|g.224 LBH_0240|m.224 type:complete len:136 (+) LBH_0240:1-408(+)	SNLIEVT	●
195	623.8531	1245.6917	2	>LBH_0313|m.289 LBH_0313|g.289 ORF LBH_0313|g.289 LBH_0313|m.289 type:complete len:277 (+) LBH_0313:1-831(+)	DIENIKITGEI	●
196	575.3201	574.3128	1	>LBH_0445|m.412 LBH_0445|g.412 ORF LBH_0445|g.412 LBH_0445|m.412 type:5prime_partial len:383 (+) LBH_0445:1-1149(+)	DKLAQ	●
197	718.3381	717.3308	1	>LBH_0445|m.412 LBH_0445|g.412 ORF LBH_0445|g.412 LBH_0445|m.412 type:5prime_partial len:383 (+) LBH_0445:1-1149(+)	GSVNDVQ	●
198	570.8166	1139.6187	2	>LBH_0494|m.460 LBH_0494|g.460 ORF LBH_0494|g.460 LBH_0494|m.460 type:complete len:668 (+) LBH_0494:1-2004(+)	TVTMLMTTIL	●

Table 2 (*Continued*)

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
199	1001.5128	1000.5055	1	>LBH_0495|m.461 LBH_0495|g.461 ORF LBH_0495|g.461 LBH_0495|m.461 type:complete len:309 (+) LBH_0495:1-927(+)		APMISAASVH		●			
200	767.4723	766.465	1	>LBH_0727|m.681 LBH_0727|g.681 ORF LBH_0727|g.681 LBH_0727|m.681 type:complete len:220 (+) LBH_0727:1-660(+)		PLLPQSL		●			
201	695.8738	1389.733	2	>LBH_0948|m.881 LBH_0948|g.881 ORF LBH_0948|g.881 LBH_0948|m.881 type:complete len:649 (+) LBH_0948:1-1947(+)		NARIQDNLYLAV		●			
202	815.4518	814.4445	1	>LBH_1022|m.953 LBH_1022|g.953 ORF LBH_1022|g.953 LBH_1022|m.953 type:complete len:565 (+) LBH_1022:1-1695(+)		DQDLVLI		●			
203	734.3551	733.3478	1	>LBH_1022|m.953 LBH_1022|g.953 ORF LBH_1022|g.953 LBH_1022|m.953 type:complete len:565 (+) LBH_1022:1-1695(+)		ENPRAF		●			
204	695.8759	1389.7372	2	>LBH_1029|m.960 LBH_1029|g.960 ORF LBH_1029|g.960 LBH_1029|m.960 type:complete len:375 (+) LBH_1029:1-1125(+)		NAGVLQDIRFKQ		●			

205	857.4269	856.4196	1	>LBH_1099|m.1026 LBH_1099|g.1026 ORF LBH_1099|g.1026 LBH_1099|m.1026 type:5prime_partial len:206 (+) LBH_1099:1-618(+)	FDPTLHQ	●
206	824.348	823.3407	1	>LBH_1154|m.1076 LBH_1154|g.1076 ORF LBH_1154|g.1076 LBH_1154|m.1076 type:5prime_partial len:554 (+) LBH_1154:1-1662(+)	DDVTEVM	●
207	944.5001	943.4928	1	>LBH_1162|m.1083 LBH_1162|g.1083 ORF LBH_1162|g.1083 LBH_1162|m.1083 type:complete len:269 (+) LBH_1162:1-807(+)	LDENDIIL	●
208	870.4579	869.4506	1	>LBH_1221|m.1131 LBH_1221|g.1131 ORF LBH_1221|g.1131 LBH_1221|m.1131 type:complete len:119 (+) LBH_1221:598-954(+)	SVAPAAAGIN	●
209	734.3551	733.3478	1	>LBH_1278|m.1190 LBH_1278|g.1190 ORF LBH_1278|g.1190 LBH_1278|m.1190 type:complete len:350 (+) LBH_1278:1-1050(+)	SRPETSG	●
210	643.3336	642.3263	1	>LBH_1471|m.1370 LBH_1471|g.1370 ORF LBH_1471|g.1370 LBH_1471|m.1370 type:complete len:327 (+) LBH_1471:1-981(+)	MVIQH	●

Table 2 (*Continued*)

No.	Mz	Mr	Peptide charges	Parent protein	Amino acid	Sequence	Extracellular	Intracellular	Reported in sequence	Reported in bioactivity	References
211	969.5253	968.518	1	>LBH_1471|m.1370 LBH_1471|g.1370 ORF LBH_1471|g.1370 LBH_1471|m.1370 type:complete len:327 (+) LBH_1471:1-981(+)		PNIMVIQH		●			
212	511.727	1021.4394	2	>LBH_1505|m.1405 LBH_1505|g.1405 ORF LBH_1505|g.1405 LBH_1505|m.1405 type:complete len:181 (+) LBH_1505:1-543(+)		NCMIGANAGV		●			
213	884.4523	883.4451	1	>LBH_1578|m.1470 LBH_1578|g.1470 ORF LBH_1578|g.1470 LBH_1578|m.1470 type:complete len:310 (+) LBH_1578:1-930(+)		GIQDPKEP		●			
214	670.4135	669.4062	1	>LBH_1627|m.1516 LBH_1627|g.1516 ORF LBH_1627|g.1516 LBH_1627|m.1516 type:5prime_partial len:329 (+) LBH_1627:1-987(+)		IPLIAGS		●			
215	853.4145	852.4072	1	>LBH_1801|m.863 LBH_1801|g.863 ORF LBH_1801|g.863 LBH_1801|m.863 type:complete len:181 (+) LBH_1801:659-1201(+)		●					

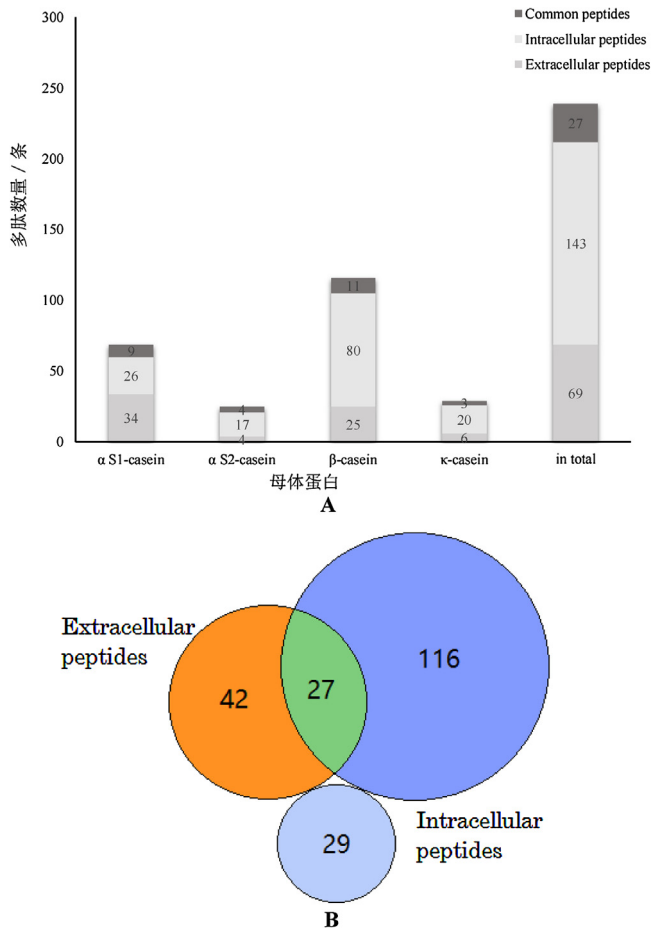


Fig. 2. Distribution of identified peptides between extracell and intracell. Numbers show the total quantity of peptides identified either in extra or in intracell in total and for all parent proteins, i.e.α_{S1}-, α_{S2}, β- and κ-casein (A), and the comparison to the peptide profile of casein fermentation in a Venn diagram (B).

extracellular peptides amino acids was 10.4, and less than or equal to 8 amino acids was 18, accounting for 26%; the average number of extracellular peptides amino acids was 10.3, and less than or equal to 8 amino acids was 40, accounting for 28%. Although the proportion of intracellular short peptides slightly larger than the extracellular peptides, on the whole, the two are basically consistent. In both, common peptides had a total of 27, just as the intersect of the orange and the blue circle in Fig. 2**B.** These peptides not only existing in extracell, but also appearing in intracell were the result of peptides generated extracellularly being transported into the cells, or result from generation in intracell and transportion to extracell again, whose way is to be further tracked and explored. However, it has been described that the bacteria can absorb the extracellular hydrolyzed peptide fragments into the cell for make use of protein in the life of metabolic activities, and then make the next utilization that most of peptides are again hydrolyzed into small molecule peptides or even a single amino acid to provide for the growth [35]. It is the found common peptides appearing in the cell that proved that it has not been degraded again and likely to be directly used by the bacteria. Z. Cheng et al. cultured lactic acid bacteria with 5-carboxyfluorescein (FAM) -labeled milk-derived peptide QEPV, made the bacteria washed three times and detected by flow cytometry, and finally the fluorescence intensity was significantly shifted, indicating that FAM-QEPV was absorbed by the cell. The qualitative and quantitative analysis by UPLC-MS/MS confirmed that QEPV could be directly absorbed from extracell to intracell for use by *Streptococcus thermophilus*. The smallest blue circle in the figure represents peptides derived from *Lactobacillus helveticus* protein, which is no coincidence on the sequence of casein-derived peptides. Whether the milk casein and the microbial protein have a such connection as same peptides or same core amino acid sequence remains to be explored.

The detected distribution of peptides amino acids in parent casein and cleavage sites of the parent casein were displayed in Fig. 3,which indicated that the released extracellular peptides and intracellular peptides are distributed unevenly among the protein sequences. In β-casein var A1, peptides amino acids are focused on 57–70 sites. And it can be found that a relative high number of peptides are released from 178 site to 209 site of β-casein var B. However, none peptides were found at position 15–37 in the sequence of β-casein. What's more, other popular distribution region are focused on the 178–199 in the sequence of α_{S1}-casein var B, 39–50 in the sequence of α_{S1}-casein var D, 72–86 and 170–182 in the sequence of κ-casein and 130–142 in the sequence of α_{S2}-casein. Cleavage in β-casein are same most, and N68–S69 in var A1 and F52–A53, V178–P179, Q182–R183, A189–F190 and L192–Y193 in var B are more easily hydrolyzed by proteases. In α_{S1}-casein, F23–F24, F179–S180, D181–I182, E189–N190, E192–K193, and L189–W199 in var B and F39–V40 in var D show a greater superiority than others. In κ-casein, only var A, A86–A87 and N181–T182 show a high susceptibility to proteolysis. And the resistance of N130–A131 and A131–V132 to hydrolysis is weaker in α_{S2}-casein. E.R.S. Kunji et al. [105] demonstrated cleavage sites are related to the specificity of some microbiological proteinases. In the case of PrtP including E group protease (PI-type) and A group protease (PIII-type), the former PI-type preferentially hydrolyzes the β-casein and the latter PIII-type is capable of hydrolyzing the α_{S1}-, β- and κ-casein, which can be further subdivided into a, b, c, d, e, f and g seven subtypes depending on different cleavage sites [106,107].

3.3. Functions confirmation and prediction of bioactive peptides

A detailed literature survey and a BIOPEP [108,109] database search were performed for the identification of known bioactive sequences in the generated peptide list in Table 2. The BIOPEP application contains a database, where sequences of biologically active peptides confirmed to have an activity either *in vivo* or *in vitro* based on literature reports are listed. A largest number of bioactive peptides are ACE inhibitors. Many research indicated that ACE can raise blood pressure by converting angiotensin I into angiotensin II, which strengthens the myocardial contractility, and at the same time makes the vascular smooth muscle contraction resulting in increased blood pressure. Consequently, ACE inhibitors can exert inhibition [110]. Twenty-one peptides were reported to have ACE-inhibitory ability. Secondly, five antioxidant peptides were identified. V.P. Shanmugam et al. [58] reported that the NAVPITPTL peptide possessed antioxidant activity of 1.49 ± 0.10 TEAC (mmol TE mg1) and significantly differed between others ($P < 0.01$). Five peptides with immunomodulation were identified. Five antibacterial peptides were reported by M. Hayes (2006) et al. [53,57,64,85,99]. Also, H. Uenishi [59] and M. J. Ojeda et al. [80] confirmed such three DPP-IV inhibitors. Dipeptidyl-peptidase 4 (DPP-4) can rapidly degrade the rising incretin in the plasma after food intake so as to hinder the synthesis of insulin and bring about a high blood glucose concentration. Therefore, DPP-4 inhibitors can ameliorate glucose tolerance [111]. Finally, there were other bioactive peptides, such as antiamnesic peptides, anticancer peptides, bitter peptides, and peptides with cell regulation ability, conducive to treat leptin-associated disorders and with lipoxygenase inhibition. Of course, there have been some peptides remained and not reported by scientists with regard to their bioactivities so far,

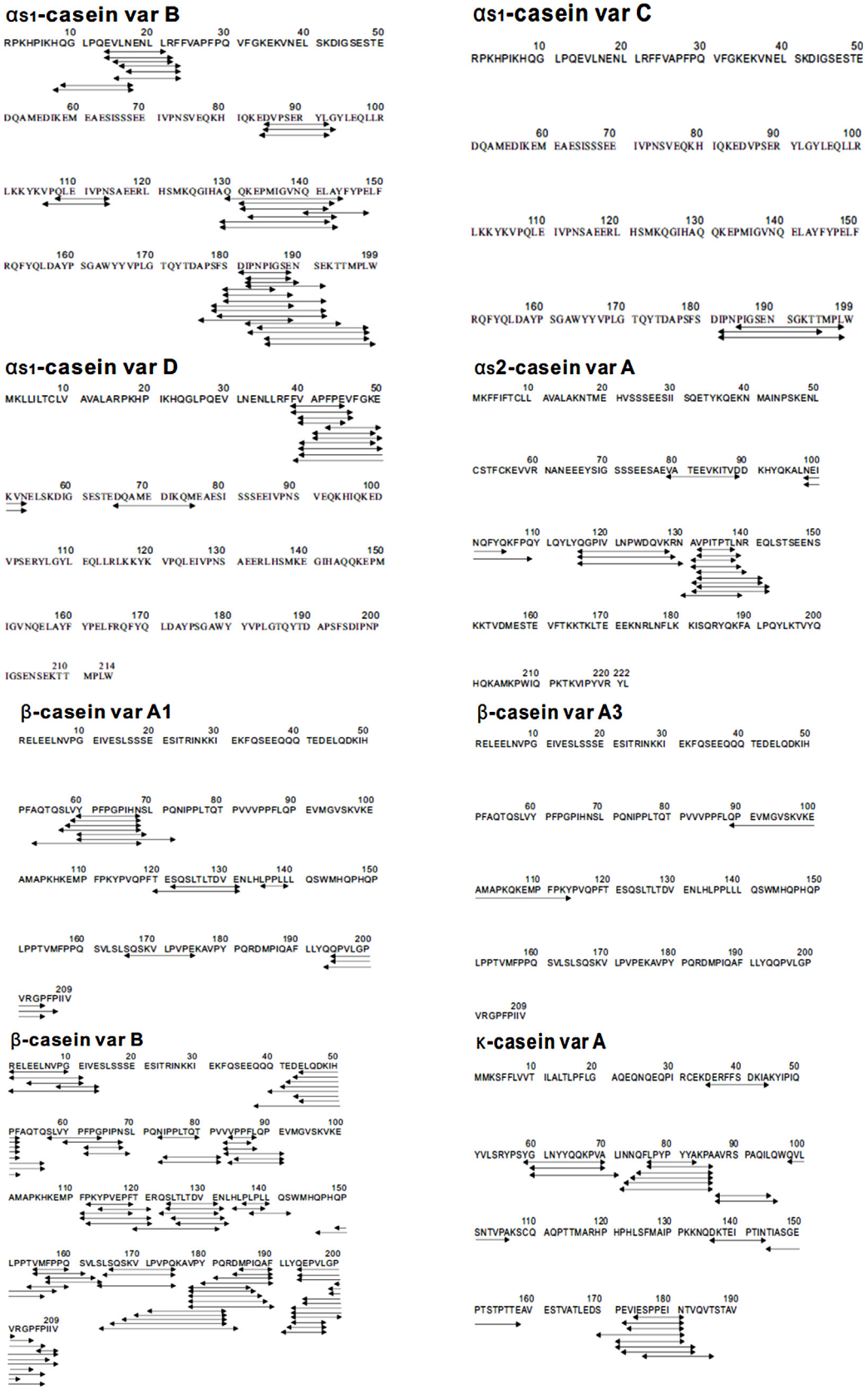
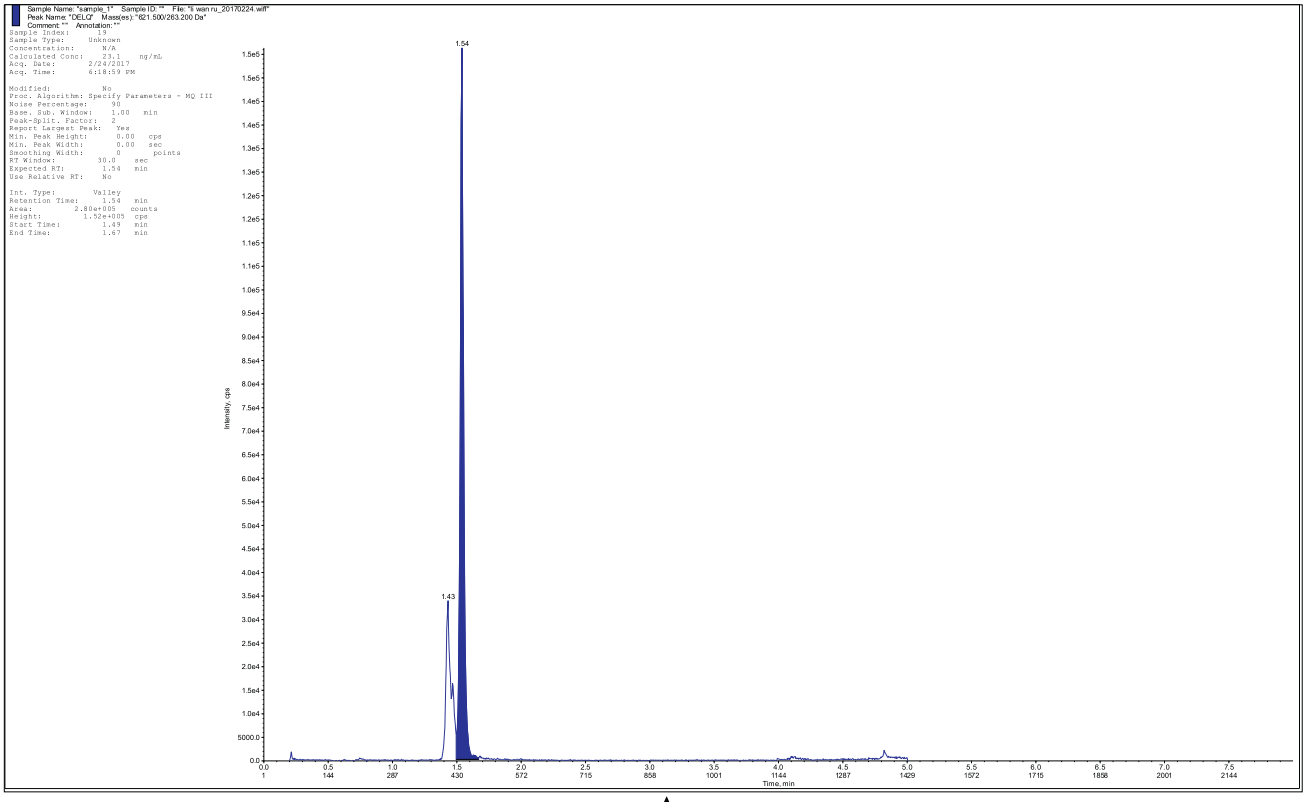
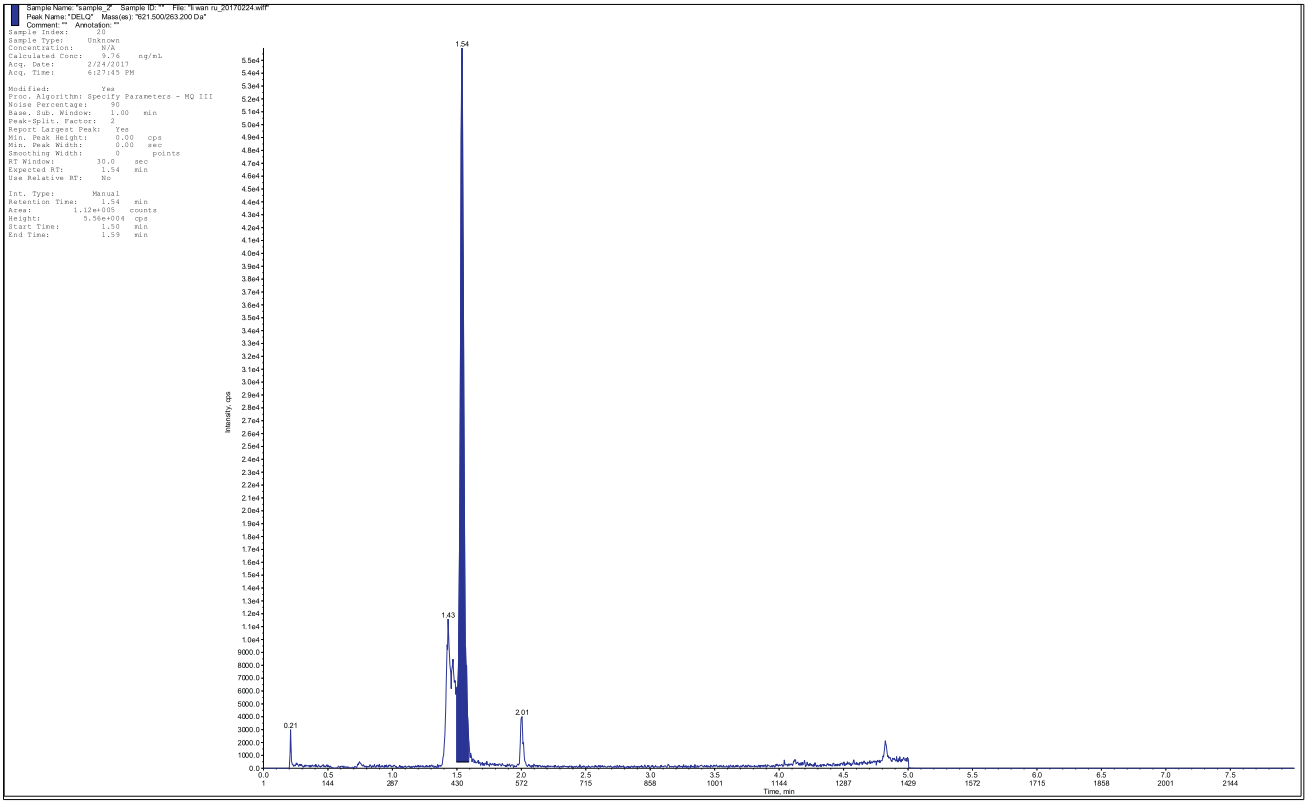


Fig. 3. Amino acid sequences of the parent proteins αS1(var B, C, D)-,αS2(var A)-,β(var A_1, A_3, B)-, andκ-casein(var A) in single letter code. Arrows represent the identified released peptides.



A



B

Fig. 4. Chromatographic peaks of DELQDKIHPF peptide in the extracellular(A) and intracellular (B) sample.

that can be called as novel peptides and could have a potential of biological activities for the human body.

Therefore all the unknown peptides were then evaluated according to bioinformatic prediction by BIOPEP and literature survey. Thirteen peptides with the potential of some biological activity are obtained. The peptide PIGSENSEKTTMPLW is a potential antihypertensive peptide because of its fragment TTMPLW proved to have a ACE-inhibitory ability [112,113]. VAPFPE possessing the features of DPP-IV inhibitory peptides (i.e., Leu, Ile or Phe at the N-terminus and/or Pro/Ala at position 2, and/or Pro at the C-terminus) according to known DPP-IV inhibitory peptides was considered to have a DPP-IV inhibitory potential [56]. B.N.P. Sah et al. [69] purified antioxidant peptides from crude peptide extract of probiotic yoghurt using ultrafiltration and reversed phase-high performance liquid chromatography, followed by GI digestion, and identified several potential antioxidant peptides including NIPPLTQT in the digestate of P19. As for the remaining 47 novel peptides, there currently are not any experimental and scientific evidence for their sequences and possible biological activities, and their acitivities and potential application value remain to be explored. Currently, most of studies on the peptide activity are focused on the antihypertensive, hypoglycemic, antioxidative, immunomodulating and other medicinal application, and more and more scientists excavate and explore bioactive peptides in the use of microbial. And our p̈eptide databaseïs the basis of our exploration of new bioactive peptides and as a database of exploring new valuable drugs through microbes or proteins in future. These new natural bioactive peptides are quite likely to be health care products for improving human health, and even become new drugs for the treatment of human diseases.

3.4. Target peptide absolute quantification

Selection of target peptide: In the Mascot search results, it was found that in a number of extracellular and intracellular peptides, a few peptides were hydrolyzed and ionized to identify more times, indirectly indicating that these peptides had a higher content and a higher ionization efficiency relative to other peptides. In this context, it was also found that there were a few peptides existing in both extracell and intracell, having the same sequences and having been identified several times. The valuable peptide most noteworthyly is DELQDKIHPF (amino acid number 43–52), which is closely related to the DELQ and the DELQDKIH found previously in our laboratory. DELQ was found to play an important role in promoting immune system activity. It was proved that the peptide has in vitro antioxidant activity by a [DPPH.] (diphenyl picryl hydrazinyl radical) method and a FRAP (Ferric Reducing Ability Power) method, and in vitro proliferating lymphocyte function by MTT method [114]. Our researchers also tested and veritied that DELQDKIH had an antioxidant activity *in vitro* using a [DPPH.] (diphenyl picryl hydrazinyl radical) method and a Total Antioxidant Capacity Assay Kit with ABTS method. Moreover, the peptide DKIHPF had been previously demonstrated to exhibit ACE inhibition with IC50 values as low as 256 M [115]. So the peptide DELQDKIHPF is not only defined as the target peptide, but also the potential bioactive peptide.

The target peptide quantification was based on the calibration curve drawn using synthesised standard peptide. The calibration curve of the developed UPLC/MRM method for DELQDKIHPF within a concentration range of 2–25 ng/mL DELQDKIHPF was obtained, of which equation fitted by linear regression was y = 1.26e+004x+-1.11e+004. Good linearity has been achieved with regression coefficient R^2 =0.9983. The acquired equation were used to quantify peptide samples according to peak areas, resulting to calculate final concentration of DELQDKIHPF peptide in both extracellular and intracellular samples. The final concentration of DELQDKIHPF was 23.1 ng/mL in extracell and 9.76 ng/mL in intracell, which laid the foundation for the subsequent research and production of bioactive peptides (Fig. 4).

So far, many studies have shown that single or several bioactive peptide fragments found from fermented dairy products have low biological activity, simple function and some even no bioactivity. The application value of single peptide may not be high. It shows that these research results are not comprehensive and lack of system. The type and quantity of bioactive peptides derived from fermented dairy products vary greatly because of the effects of fermentation conditions, species of lactic acid bacteria and substrate concentration. In terms of functional characteristics, it is likely a series of peptides that play a role in the bioactivity as a comprehensive result; In terms of yield, the yield of single peptide in production applications is not high, and it can be said to be low, but the total yield of the mixed peptides is relatively higher. Therefore, in the future production of peptide drugs or health care products, it is combined peptides or mixed peptides that have higher value, more practical and greater prospects for development in the market in which health industry as the mainstream.

4. Conclusion

In this study, *Lactobacillus helveticus CICC6024* was used to efficiently ferment milk-casein under relatively fixed fermentation conditions in order to achieve efficient production of bioactive peptides. 241 bioactive peptides from the extracell and intracell of the fermentation broth were isolated and identificated by a series steps of centrifugation, ultrasonic cell disruption, ultrafiltration, solid phase extraction, gastrointestinal simulated digestion and NanoLC-Q-TOFMS. The comprehensive analysis of the peptide profile and the summary of their functions were carried out. A specific p̈eptide databaseÏike a large v̈aultẅere published, where it was the types and quantities of bioactive peptides derived from casein and probiotics and their various biological activities that were explored, the potential application of extracellular and intracellular peptides also done. Also 47 novel bioactive peptides derived from casein were found, which laid the foundation for following investigation of new bioactive peptides. Finally, one of the potential peptide DELQDKIHPF was quantitatively analyzed by UPLC-U3Q method. The present work provides useful information that could be used as a basis of subsequent exploration and indicators for various production parameters of peptides capable of treating disease and protecting health, which may have a beneficial influence on human health industry.

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