

**Bioactive hydrolysates from casein: Generation,
identification, and *in silico* toxicity and allergenicity
prediction of peptides**

Running Title: Identification and Prediction of Bioactive Peptides from Casein

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ABSTRACT

BACKGROUND: Bioactive casein peptides have attracted considerable attention for their applications in industry. However, there is little clarity regarding mass spectrometric profiles for peptides in enzymatic hydrolysates of casein produced under varying conditions. In this study, the compositions of the peptides from casein hydrolysates were compared for different E/S and hydrolysis times. The toxicity, allergenicity and bioactivity of the identified peptides were assessed *in silico*.

RESULTS: A total of 70 unique peptides were identified, and there were 28, 21, 13 and 8 peptides from α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein, respectively. The

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peptide number decreased with the increase in E/S and hydrolysis time. Moreover, peptides with molecular mass (M_r) ranging from 1000 to 1500 Da occupied the highest proportion of 31.43%, and almost all of the peptides showed relative M_r less than 5000 Da. *In silico* analysis showed that all of the peptides were non-toxic and non-allergenic, and several of them were assessed by PeptideRanker as having a relatively high likelihood of being bioactive peptides.

CONCLUSIONS: Composition of the peptides in the casein hydrolysates varied with the enzymolysis conditions. This study's results may facilitate the production of target bioactive peptides by controlling E/S and hydrolysis time, which is beneficial for the application of casein peptides in the functional food industry.

Keywords: Casein peptides; Enzymatic hydrolysis; Identification; UPLC-Q-TOF-MS/MS; *In silico* analysis

INTRODUCTION

Milk has attracted attention in human daily diet as a particularly good source of high quality protein with important biological functions.^{1,2,3} Bovine casein is a major protein (80%) in milk and includes α_{s1} -casein (α_{s1} -CN), α_{s2} -casein (α_{s2} -CN), β -casein (β -CN) and κ -casein (κ -CN). Casein is widely used as a functional ingredient in food products due to its nutritional merits.^{4,5,6} Casein has been considered to be a natural vehicle for bioactivities in drug delivery systems because of its particular structure and physicochemical properties.^{7,8} Moreover, casein releases bioactive peptides with multiple activities of promoting health and preventing disease, such as antioxidant, antibacterial, anticoagulant, immunomodulatory, and ACE inhibitory (ACEI) activity, through enzymatic hydrolysis, microbial fermentation, and other combined

mechanisms.^{9,10,11} Two types of ACEI peptides were released by neutral protease hydrolysis of κ -casein: RYPSYG (fragment 25-30) and DERF (fragment 15-18).¹² The following peptides were identified from fermented milk product: two antihypertensive peptides, LHLPLP (fragment 133-138) and LVYFPFGPIPNSLPQNIPP (fragment 58-76) from β -casein and one peptide (YQEPVLGPVRGPFPIIV) from β -casein (fragment 193-209) with the activities of ACE inhibition and anticoagulant activities.^{13,14} Two long peptides, the N-terminal of α _{s1}-casein and kappa-caseinoglycopeptide, were detected in plasma after human gastrointestinal digestion,¹⁵ which indicated that the bioactive peptides released from food proteins may be directly absorbed in the gastrointestinal environment. Enzymatic hydrolysis is generally used for producing bioactive peptides *in vitro*, and several factors, such as enzyme, substrate concentration and temperature, produce hydrolysates with varied activities due to different peptide compositions.¹⁶

Although bioactive peptides are important in the medical and food industry, there is a lack of systematic methods for producing bioactive peptides from food-derived proteins. Commercial milk-derived bioactive peptides remain limited due to lack of appropriate large-scale technologies for production.⁹ Recently, the emergence of mass spectrometry has improved analysis of peptide mixtures during proteolytic degradation.¹⁷

Toxicity, allergenicity and bioactivity are three vital important indexes for producing bioactive peptides and related products. Because the traditional methods for determining those indexes are complex and time-consuming, the *in silico* method is

considered to be an effective and practical method to predict the toxicity, allergenicity and bioactivity of peptides. The recognized *in silico* tools for predicting the toxicity, allergenicity and bioactivity are ToxinPred, AlgPred and PeptideRanker, respectively.^{18,19,20} These tools have improved the technology and has aided production of food-derived functional peptides.

The purpose of this study was to explore the profiles of peptides derived from casein hydrolysis under various conditions. The peptidome of casein hydrolysates under varied conditions of trypsin hydrolysis was analyzed using UPLC-Q-TOF-MS/MS. Additionally, the relations between the peptide composition of the casein hydrolysates and the hydrolysis conditions were investigated, and toxicity, allergenicity and bioactivity were predicted *in silico* to facilitate production of bioactive peptides from bovine casein using enzymatic technology.

MATERIALS AND METHODS

Materials and chemicals

Bovine casein and trypsin (EC 3.4.21.5) were purchased from Solarbio (Beijing, China). Acetonitrile (ACN, HPLC grade) was supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). Formic acid (FA) was from Fluka (Buchs, Germany), and low range protein marker (1.7-40 kDa) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation of casein hydrolysates

Bovine casein (4%, w/v) was dissolved in Tris-HCl buffer (50 mM, pH 8.0). Hydrolysis was initiated by adding different amounts of trypsin, *i.e.*, the ratio of trypsin to casein is 1000 u g⁻¹ (H1K), 2000 u g⁻¹ (H2K), 3000 u g⁻¹ (H3K), 4000 u g⁻¹ (H4K) and 5000 u g⁻¹ (H5K), respectively. Enzymatic hydrolysis was conducted at 37 °C for 30~240 min (named as H30, H60, H120, H180 and H240), and the pH was maintained at 8.0 using 50 mM tris base solution. The hydrolysis was terminated by heating the samples in a 100 °C water bath for 10 min. The samples were centrifuged at 7000×g (4 °C) for 20 min after the temperature was reduced to less than 25 °C, and the supernatants were obtained and stored at -80 °C before analysis.

Determination of hydrolysis degree

The method described by Adler-Nissen at 1986²¹ was adopted to determine the degree of hydrolysis (DH). During hydrolysis, the pH (8.0) was maintained by using 0.2 M NaOH, and the amount of NaOH consumed was recorded to calculate the DH by means of the following equation (1),

$$DH(\%) = \frac{h}{h_{\text{tot}}} \times 100 = \frac{B \times Nb}{M_p} \times \frac{1}{\alpha} \times \frac{1}{h_{\text{tot}}} \times 100 \quad (1)$$

where Nb is the normality of NaOH (0.2 M); B is the consumed volume (mL) of NaOH; h_{tot} indicates the total number of peptide bonds in the protein, assumed to be 8.2 meq/g in casein; and M_p represents the mass (g) of the protein.²¹ α indicates the average degree of dissociation of the α -NH₂ groups in the protein substrate, expressed in the following equation (2), where pH and pK depend on the hydrolysis situation.

$$\alpha = \frac{10^{\text{pH}-\text{pK}}}{1+10^{\text{pH}-\text{pK}}} \quad (2)$$

Tricine-SDS-PAGE

Supernatants from centrifugation were homogenized and diluted by Milli-Q water from a Milli-Q system (Millipore, Massachusetts, USA), and 10 μ L of the samples were mixed with 10 μ L loading buffer (100 mM Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-Mercaptoethanol and 0.2% bromophenol blue). Subsequently, the samples were heated for 10 min at 100 °C before loading them onto a gel containing 16% separating gel and 4% stacking gel, configured according to the method reported by Schagger.²² Electrophoresis was conducted at a constant voltage of 120 V by using a BIO-RAD Mini-PROTEAN® 3 cell System (Bio-Rad Laboratories Ltd., Hertfordshire, UK).

Identification of the peptides by UPLC-Q-TOF-MS/MS

First, 0.5 mL of supernatants of casein hydrolysates was transferred to a 1.5 mL Eppendorf tube for vacuum freeze drying (ALPHA 1-4 LSC, Martin Christ, Osterode, Germany) and subsequent re-dissolved using 0.5 mL FA aqueous solution (0.1%, v/v). All samples were filtered using 0.22 μ m micro pore membrane before mass spectrometric analysis. The samples were analyzed by an ultrahigh-performance liquid chromatography (UPLC) (Thermo Finnigan, San Jose, CA, USA) coupled to a Microtof-II (Bruker Daltonik, Bremen, Germany). The chromatographic system was equipped with a 150 mm $\times$ 3.0 mm C18 column (Phenomenex, Torrance, California, USA) with a particle size of 3.0 μ m. Mobile phase A and B were 0.1% FA (v/v) in water and in ACN, respectively. The analysis was run at 30 °C with a flow rate of 0.4 mLmin⁻¹, and the injection volume was 10 μ L. For the separation of the peptides, an LC elution program was set for a total of 70 min. The elution program consisted of 5%

B for 5 min, 30 min gradient elution from 5% B to 35% B and maintained for 5 min, 2 min gradient elution from 35% B to 80% B with 80% B maintained for 13 min and 1 min gradient elution from 80% B to 5% B and maintained for 14 min. The first 5 min of elution was performed to elute out salts and other impurities. The eluent from this step was considered as waste and was not utilized for mass analysis. The parameters of MS/MS analysis were set as positive ion mode; 200 °C dry temperature, 50-2200 m/z mass range and the voltage of capillary at 4.5 kV. The top ten intense precursors for subsequent fragmentation of automatic data-dependent acquisition mode was employed to analyze all the samples.

Data analysis by Mascot was conducted by downloading the *Bos Taurus* database (July, 2016) from NCBI (<https://www.ncbi.nlm.nih.gov/>) and searching using Mascot 2.5.1.0 (Matrix Science, London, UK). The data were searched using Proteinscape software (Bruker Daltonik, Bremen, Germany) in the *Bos Taurus* database. The searching parameters were set as follows: below 1% of the false-positive discovery rate (FDR) of peptide identification; 0.03 Da of the mass/mass tolerances; 10 ppm of the peptide tolerances; a maximum of 2 missed cleavages. It is necessary for an identified protein that at least one unique peptide was identified and confirmed.

***In silico* predictions of peptide toxicity, allergenicity and bioactivity**

The peptides identified were evaluated for potential toxicity *in silico* using ToxinPred, available at <http://www.imtech.res.in/raghava/toxinpred/>.¹⁸ The peptides were additionally evaluated for potential allergenicity using AlgPred, an online tool

available at <http://www.imtech.res.in/raghava/algpred/>. Two modules were used: the SVM module based on amino acid composition prediction and the MEME/MAST motif prediction approach.^{19,23} In addition, the likelihood that the peptides are bioactive was determined by PeptideRanker, a server for the prediction of bioactive peptides based on a novel N-to-1 neural network, which is available at http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php.²⁰

PeptideRanker ranked all peptides by giving scores ranging from 0 to 1 of a certain peptide, and the higher of the scores, the greater likelihood of the peptide to be bioactive.²⁰ In the present study, the threshold of the PeptideRanker score was set as 0.5, which indicates that any peptide with predicted scores exceeding this threshold was considered to be more likely to be bioactive.

RESULTS AND DISCUSSION

DH changing with different hydrolysis conditions

In this study, 5 different concentrations of trypsin and 5 different hydrolysis times were designed to study the effect of hydrolysis conditions on the peptide composition of casein hydrolysates at 37 °C and pH 8.0. Fig. 1 shows that the DH increased with increase in trypsin application for the same hydrolysis time in general. Additionally, DH increased with increased hydrolysis time at the same trypsin concentration. Furthermore, the DH increased rapidly in the initial 20 min, showing a trend similar to that observed in a previous study.²⁴ The DH rose from 20 min to 90 min during the hydrolysis process. Finally, the curves rose slowly and tended to be constant at the end of the hydrolysis course. According to the study of Adler-Nissen,²¹

the peptides released in a hydrolysis system may inhibit the enzyme activity, which may explain the changing patterns of the DH.

As shown in Fig. 1, the sample with an enzyme concentration of 5000 u g⁻¹ (H5K) showed the highest DH of 18.37%±0.29 at the end of hydrolysis of 240 min, whereas the H1K group demonstrated the lowest DH of 5.76%±0.35. Mora reported that the enzyme concentration in the hydrolysis system normally and the substrate concentration are vital factors.²⁵ It was reported that limited protein hydrolysis (DH < 10%) often produce hydrolysates that differ in functional properties, whereas extensive hydrolysis is beneficial to obtain bioactive peptides. However, over-hydrolysis may cause enzymatic degradation of the bioactive peptides, resulting in loss of bioactive properties. In this aspect, the hydrolysis degree of the hydrolysates in the present study may result in more bioactive peptides.

Electrophoretic profiles of the casein hydrolysates

Enzymatic hydrolysis can break the peptide bonds in casein molecules. The compositions of the peptides released in hydrolysates varied significantly depending on the different conditions in the enzymolysis system. As a result, the hydrolysates exhibited varying bioactivities due to different peptide compositions, which makes it important to optimize the hydrolysis parameters to prepare peptides with a target bioactivity.

DH and SDS-PAGE provided two indexes of the degradation extent of casein molecules in the course of hydrolysis. As shown in Fig. 2-(a), (b) and (c), the effects of E/S on the hydrolysis process were tested in Tricine-SDS-PAGE. The results

showed that there were no intact casein molecules in each sample at the end of hydrolysis, and the DH increased significantly with increase in enzyme application and the reaction time, similar to the trends observed with the DH curve in Fig. 1. In addition, Fig. 2-(a) demonstrated that the relative molecular mass (M_r) of the peptides in the hydrolysate primarily ranged from 5 KDa to 10 KDa. Increase in hydrolysis time increased production of peptides with M_r approximately 5 KDa rather than 10 KDa. Furthermore, as shown in Fig. 2-(c), the hydrolysates from H5K group after 180 min showed no electrophoretic band in Tricine-SDS-PAGE, indicating that several peptides with low M_r were produced, which could not be observed in the SDS-PAGE.

Hydrolysis time is another important factor for producing bioactive peptides in the course of enzymolysis. As shown in Fig. 2-(d), when the hydrolysis time increased to 30 min, the M_r of peptides in all the hydrolysates (1000 to 5000 u g^{-1}) ranged from 5 KDa to 10 KDa. In Fig. 2-(e), it was observed that few peptides were left in the H4K group when the hydrolysis time was more than 180 min. No peptides were observed when the E/S increased to 5000 u g^{-1} in the gel.

To intensively analyze the peptide compositions of hydrolysates (1000 to 5000 u g^{-1} , 240 min), especially for those peptides with relatively lower content and lower M_r , 2 times the amount of sample were applied in the Tricine-SDS-PAGE, with the results shown in Fig. 2-(f). It was found that almost all peptides distributed at the field of peptides with M_r of approximately 1.7 KDa. Tricine-SDS-PAGE indicated that the DH of the samples related to the hydrolysis and amount of enzyme. When casein

was hydrolyzed by trypsin completely, the compositions of the hydrolysates were similar due to the limitation of enzymatic hydrolysis in the system, which may be observed because trypsin has restrictive cleavage sites at Lys (K) and Arg (R).^{26,27}

Peptides identification by UPLC-Q-TOF-MS/MS

UPLC-Q-TOF-MS/MS is widely used in peptide identification due to its high sensitivity and stability.²⁸ In the present study, the peptide compositions of these casein hydrolysates prepared by different hydrolysis parameters were analyzed and compared. As shown in Table 1, totally 70 unique peptides have been identified from all hydrolysates. The length of those peptides ranged from 7 to 56 amino acids. Among those peptides, 28 were from α_{s1} -CN, 21 from α_{s2} -CN, 13 from β -CN and 8 from κ -CN, as shown in Fig. 3. Compared with three other kinds of casein molecules, few peptides have been identified from κ -casein, possibly because the content of κ -CN (11.9%) in casein micelles was less than α_{s1} -CN (35.6%) and β -CN (33.6%)²⁹ and the cleavage sites of trypsin, Lys and Arg in κ -CN (14 sites) was less than α_{s1} -CN (21 sites), α_{s2} -CN (31 sites) and β -CN (16 sites).³⁰

Fig. 4 shows the overlay of the peptides identified from α_{s1} -CN, α_{s2} -CN, β -CN and κ -CN. There were 127, 132, 205, and 72 amino acids residues in the peptides identified in α_{s1} -CN (199 AA), α_{s2} -CN (207 AA), β -CN (209 AA) and κ -CN (169 AA), respectively. The coverage rate of identified peptides of casein molecules ranged from 42.6% to 98.1%, and β -CN showed the highest coverage rate of 98.1%. These results indicated that β -CN was advantageous for producing bioactive peptides by enzymatic

hydrolysis compared with other casein molecules because more bioactive peptides have been identified from β -CN than from α_{s1} -CN, α_{s2} -CN and κ -CN.³⁰

Effects of enzymatic hydrolysis conditions on release of peptides

As shown in Fig. 5, the Mr. distribution of these 70 identified peptides were investigated. Peptides with Mr. of 1000-1500 Da formed the highest proportions at 31.43%. The proportions of peptides with Mr. <1000 Da and 1500-2000 Da were the same (21.43%). Peptides with Mr. of 2000-3000 Da accounted for 15.71%. However, only 10% peptides with Mr. >3000 Da were identified. One peptide with an Mr. of 6358.2487 Da was identified in the H1K group hydrolyzed for 30 min and 60 min. Moreover, according to the limit of Mascot searching, only those peptides consisting of more than 4 amino acid residues can be identified. The peptides with Mr. less than 500 Da have not been identified and involved in the present study.

Fig. 6-(a) shows the Venn diagram of casein peptides identified under the conditions of different E/S ratios. A total of 65, 52, 53, 49 and 44 peptides were observed for E/S ratios of 1000 u g⁻¹ (1K), 2000 u g⁻¹ (2K), 3000 u g⁻¹ (3K), 4000 u g⁻¹ (4K) and 5000 u g⁻¹ (5K), respectively. It was observed that 35 peptides existed in all 5 enzyme concentrations. Moreover, 12 unique peptides were identified in the H1K group. In contrast, only 1 unique peptide was identified in H3K and H4K, and there was no unique peptide in H2K and H5K. Fig. 6-(b) shows a Venn diagram of casein peptides identified for different hydrolysis times with varying enzyme amounts. A total of 58, 60, 53, 49 and 50 peptides were identified in the hydrolysates of different hydrolysis time of 30~240 min, respectively. Thirty-six peptides were

co-present in above five hydrolysis times. Furthermore, with the increase of hydrolysis time, the number of unique peptides identified in these hydrolysates decreased accordingly; there were 4, 5, 3, and 1 unique peptide in H30, H60, H120 and H180, respectively, and there was no unique peptide in H240. Seven peptides from H120 overlapped with H30, H60 and H180. However, only 1 peptide from H240 overlapped with H30, H120 and H180. In general, there were no significant differences in the peptides identified from the 5 groups when skipping the volume of trypsin. Fig. 7 shows the relation between the peptides released, the enzyme amount and hydrolysis time. The data are shown in Table 2. Following an enzyme application of $1000 \mu\text{g}^{-1}$ and hydrolysis for 60 min, up to 53 peptides were identified. In general, few peptides were identified when the hydrolysis time was prolonged with the amount of enzyme remaining constant, which may be because the hydrolysates comprised of completely and partly hydrolyzed peptides at the original stage of the hydrolysis and more peptides were consequently left in the hydrolysates. However, some of the large peptides that have not been completely broken continued to be cleaved thoroughly when hydrolysis time was extended, and the number of peptides were consequently reduced. Meanwhile, when the hydrolysis time was constant, the number of peptides decreased with increase in enzyme application, possibly because the DH increases with the amount of enzyme in the same hydrolysis time, as indicated in Fig. 1. Similar conclusion has been drawn by other studies indicating that the increase of DH leads to a decrease in the number of peptides to a certain extent.^{31, 32, 33}

Toxicity, allergenicity and bioactivity of identified peptides

The peptides were assessed for potential toxicity using the ToxinPred based on the SVM (TrEMBL) method.¹⁸ Results showed that all casein hydrolysates were non-toxic, and no peptides with potential toxicity were identified. The potential allergenicity of peptides identified was assessed *in silico* using AlgPred with two typical modules, SVM and MEME/MAST. The SVM module performs predictions based on amino acid composition. MEME and MAST are tools for discovering motifs in a set of related protein sequences and for finding biological sequence database for sequences that include at least a group of known motifs, respectively.¹⁹ AlgPred has a limitation that only those peptides longer than ten amino acids in length can be analyzed, so that the data on potential allergenicity of shorter peptides were omitted. As shown in Table 1, 37 peptides and 4 peptides from casein hydrolysates were predicted to be allergens or potential allergens by using the SVM module. However, all of the casein hydrolysates peptides were predicted to be non-allergens using the MEME/MAST module. Potential allergenicity of short peptides, *i.e.*, the less than 10 amino acids in length, was not available by both of the modules. It is well known that the allergenic activity of peptide is closely related to its length.^{34, 35} According to the MEME/MAST predictive module, the peptides showed no allergenicity, which provided the allergic possibility of these peptides, and the allergic reactions to human bodies need be investigated by *in vivo* experiments.

Seventy different peptides derived from casein hydrolysates were identified, and their activities were predicted by PeptideRanker, an *in silico* tool to predict the likelihood that the peptides are bioactive among a set of peptides. The higher the

PeptideRanker score, the greater the likelihood of peptide bioactivity. As shown in Table 1, the highest PeptideRanker score is 0.89, and multiple peptides from casein hydrolysates showed relatively high PeptideRanker scores of 22 peptides over 0.5, which indicates that these peptides were predicted to be bioactive with high possibility. In the casein hydrolysis, 15 peptides have been reported to be bioactive by comparing the data in the BIOPEP database and those related literatures published in Web of Science. Among these 15 peptides, 9 peptides showing ACEI activity are FFVAPFPEVFGK (0.86), PFPEVFGK (0.72), ALNEINQFYQK (0.20), FALPQYLK (0.66), NMAINPSK (0.21), AMKPWIQPK (0.62), AVPYPQR (0.57), GPFPIIV (0.81) and YIPIQYVLSR (0.28); five peptides with antioxidative activity include EDVP SER (0.1), FALPQYLK, AVPYPQR, VLPVPQK (0.20) and VKEAMAPK (0.14). FFVAPFPEVFGK, DMPIQAFLLYQEPVLGPVR (0.55), RELEELNVPGEIVESLSSEESITR (0.09) and MAIPPKK (0.51) were reported to possess anticancer, anti-inflammatory, immunomodulating and antithrombotic activities. Moreover, YIPIQYVLSR was reported to be a peptide with immunomodulating and opioid antagonist activities. In addition, a total of 55 peptides without any reports about their bioactivity were identified in the present study. The PeptideRanker scores of 14 peptides in those novel peptides were proved to be higher than 0.5, which indicated that these peptides may be potential multiple bioactive peptides. Among these bioactive peptides, 7 peptides showed lower PeptideRanker scores (below 0.5). Thus, it is obvious that the peptides with lower PeptideRanker scores can also be bioactive. Therefore, the PeptideRanker scores of the peptides only

represent the likelihood of a peptide to be bioactive. Based on the assessment of toxicity, allergenicity and bioactivity of the identified peptides, the peptides derived from casein hydrolysates may be safe and may exhibit application potential in the functional food industry. The *in silico* prediction tools (ToxinPred, AlgPred, PeptideRanker) have the advantages of rapid and no cost. They provide a theoretical basis for further research to discover potential bioactive peptides from functional ingredients. Therefore, the activity of peptides should be confirmed by *in vitro* and *in vivo* tests.

CONCLUSIONS

Seventy unique peptides were released and identified from casein hydrolysates with different DH in the present study using UPLC/Q-TOF-MS/MS, and there were 28, 21, 13 and 8 peptides from α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein, respectively. Moreover, the number of peptides in the hydrolysates decreased with increase in enzyme amount and hydrolysis time. Peptides with Mr. ranging from 1000 to 1500 Da formed the highest proportion at 31.43%, and almost all peptides showed relatively low Mr. of less than 5000 Da. These hydrolysates consisted of peptides that were predicted to be non-toxic and non-allergenic and were assessed for bioactivity. The results indicated that different peptides generated by trypsin digestion under different conditions may promote the development of directed enzymolysis technologies and may show the potential application of casein hydrolysates as bioactive ingredients in the functional food industry.

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Table 1. Identification and prediction of peptides from casein hydrolysates released by trypsin digestion.

No.	Molecular weight	L.E.N	Sequence	Range	Protein	Can be identified	Peptide ranker	Toxicity	Allergenicity		Activity
									M E M E/ M A S T	S V M	
1	1758.9377	15	HQGLPQEVLENLLR	8-22	α_s 1 ⁻ C N	All	0.54	Non	Non	Allergen	Part
2	1579.8206	14	VPQLEIVPNSAEER	106-119	α_s 1 ⁻ C N	All	0.19	Non	Non	Allergen	/
3	1870.9788	16	YKVPQLEIVPNSAEER	104-119	α_s 1 ⁻ C N	All	0.20	Non	Non	Allergen	/
4	1336.6735	11	HIQKEDVPSEER	80-90	α_s 1 ⁻ C N	All	0.10	Non	Non	Allergen	/
5	830.3770	7	EDVPSEER	84-90	α_s 1 ⁻ C N	All	0.10	Non	-	-	Antioxidative ³⁶
6	945.5131	8	EKVNELSK	35-42	α_s 1 ⁻ C N	A0.5, B, C0.5-C3, E	0.04	Non	-	-	/

7	13 83. 72 27	1 2	FFVAPFPEVFGK	23 -3 4	α_s 1 ⁻ C N	All	0. 86	N on	N on	Al ler ge n	ACE inhibit or; ³⁷ Antica ncer; ³⁸
8	12 66. 69 72	1 0	YLGYLEQLLR	91 -1 00	α_s 1 ⁻ C N	All	0. 35	N on	-	-	/
9	16 40. 86 03	1 4	FFVAPFPEVFGKEK	23 -3 6	α_s 1 ⁻ C N	A0.5,A1, B0.5, B1, C0.5, E0.5	0. 65	N on	N on	Al ler ge n	/
10	23 15. 12 96	1 9	EPMIGVNQELAYFYPEL R	13 3- 15 1	α_s 1 ⁻ C N	A, B0.5-B3, C0.5, C1, D0.5, D1, E0.5, E1	0. 36	N on	N on	Al ler ge n	/
11	20 79. 06 36	1 7	EDVPSERYLGYLEQLLR	84 -1 00	α_s 1 ⁻ C N	A0.5	0. 26	N on	N on	Al ler ge n	/
12	15 05. 77 73	1 3	LHSMKEGIHAQQK	12 0- 13 2	α_s 1 ⁻ C N	A0.5, B0.5, B1, D0.5	0. 09	N on	N on	Al ler ge n	/
13	22 34. 22 83	1 9	HPIKHQGLPQEVLENLL R	4- 22	α_s 1 ⁻ C N	A, B0.5, B2, B3, C0.5, C1, C2,C4, D0.5, D1, D2	0. 58	N on	N on	Al ler ge n	Part
14	24 67. 28 93	2 1	YKVPQLEIVPNSAEERLH SMK	10 4- 12 4	α_s 1 ⁻ C N	A1	0. 06	N on	N on	N on	/

15	199.0738	17	KYKVPQLEIVPNSAEER	103-119	α_s 1 ⁻ CN	A1-A3, D0.5	0.21	N on	N on	Al ler ge n	/
16	2097.1694	18	PIKHQGLPQEVLENENLLR	5-22	α_s 1 ⁻ CN	A1, A3, B1	0.61	N on	N on	Al ler ge n	Part
17	3802.8963	32	LHSMKEGIHAQQKEPMIG VNQELAYFYPELFR	120-151	α_s 1 ⁻ CN	A1	0.05	N on	N on	Po te nti al Al ler ge n	/
18	3206.5859	27	EGIHAQQKEPMIGVNQEL AYFYPELFR	125-151	α_s 1 ⁻ CN	A1-A4, B0.5, B2, C0.5, C1, D0.5, D1	0.16	N on	N on	Po te nti al Al ler ge n	/
19	1707.9155	15	KVPQLEIVPNSAEER	105-119	α_s 1 ⁻ CN	A1-A4, B0.5, B1, B2, B4, C0.5, C1, C4, D0.5-D2, D4, E	0.24	N on	N on	Al ler ge n	/

20	35 52. 75 33	30	SMKEGIHAQQKEPMIGVN QELAYFYPELFR	12 2- 15 1	α_s 1- C N	A1	0. 12	N on	N on	Po te nti al Al ler ge n	/
21	14 93. 79 19	13	FVAPFPEVFGKEK	24 -3 6	α_s 1- C N	A1, B0.5	0. 61	N on	N on	Al ler ge n	/
22	12 36. 65 43	11	FVAPFPEVFGK	24 -3 4	α_s 1- C N	A1-A3, B0.5, C0.5, C3, C4, E1, E2	0. 84	N on	N on	Al ler ge n	Part
23	17 66. 75 16	16	DIGSESTEDQAMEDIK	43 -5 8	α_s 1- C N	A2, A4, B0.5, B2-B4,C, D, E	0. 08	N on	N on	Al ler ge n	/
24	12 04. 59 17	9	AYFYPELFR	14 3- 15 1	α_s 1- C N	A2, D0.5	0. 89	N on	-	-	/
25	10 98. 60 33	9	EVLNENLLR	14 -2 2	α_s 1- C N	A2, A3, B2, B4, C3, C4, D2-D4, E0.5, E3, E4	0. 19	N on	-	-	Part
26	11 78. 51 25	10	TEDQAMEDIK	49 -5 8	α_s 1- C N	B3, C4, D1, E1	0. 07	N on	-	-	/

27	94.82	8	DQAMEDIK	51-58	α_s 1 ⁻ C N	C2	0.16	N on	-	-	/
28	91.94804	8	PFPEVFGK	27-34	α_s 1 ⁻ C N	D2	0.72	N on	-	-	ACE inhibit or; ³⁹
29	1245.6353	10	ITVDDKHYQK	71-80	α_s 2 ⁻ C N	A11	0.08	N on	-		/
30	1194.6721	11	NAVPITPTLNR	11-125	α_s 2 ⁻ C N	A11	0.22	N on	N on	Al ler ge n	/
31	1366.6881	11	ALNEINQFYQK	81-91	α_s 2 ⁻ C N	A, B, C, D, E0.5-E3	0.20	N on	N on	Al ler ge n	ACE inhibit or; ⁴⁰
32	978.5538	8	FALPQYLK	17-181	α_s 2 ⁻ C N	A11	0.66	N on	-	-	ACE inhibit or; ⁴⁰ Antiox idative ; ⁴¹
33	1350.7732	12	RNAVPTPTLNR	11-125	α_s 2 ⁻ C N	A11	0.18	N on	N on	Al ler ge n	/
34	873.4378	8	NMAINPSK	25-32	α_s 2 ⁻ C N	A11	0.21	N on	-	-	ACE inhibit or; ⁴⁰
35	1031.6240	8	NRLNFLKK	15-166	α_s 2 ⁻ C N	A0.5-A3, B0.5, B1	0.43	N on	-	-	Part

36	12 46. 65 17	1 0	TKLTEEEKNR	15 1- 16 0	α_s 2- C N	A0.5, B0.5, B1, B3, B4, C0.5-C3, D0.5, D1, D3, E0.5	0. 03	N on	-	-	Part
37	97 4.5 91 3	8	TKVIPYVR	19 8- 20 5	α_s 2- C N	A0.5-A2, B0.5, C0.5, D0.5	0. 13	N on	-	-	Part
38	97 6.5 07 7	8	TKLTEEEK	15 1- 15 8	α_s 2- C N	A0.5, B0.5, B1, B3, B4, C3, D3	0. 03	N on	-		Part
39	15 13. 73 34	1 3	TVDMESTEVEFTKK	13 8- 15 0	α_s 2- C N	A0.5-A2, B0.5,B1, C0.5,D0.5	0. 06	N on	N on	Al ler ge n	/
40	10 97. 60 55	9	AMKPWIQPK	18 9- 19 7	α_s 2- C N	A, B0.5-B2, C0.5, C1, D0.5, D1, E0.5	0. 62	N on	-	-	ACE inhibit or; ⁴²
41	13 85. 63 85	1 2	TVDMESTEVEFTK	13 8- 14 9	α_s 2- C N	A, B, C, D0.5-D3, E0.5-E2	0. 06	N on	N on	Al ler ge n	/
42	13 97. 77 07	1 1	YQKFALPQYLK	17 1- 18 1	α_s 2- C N	A0.5	0. 51	N on	N on	Al ler ge n	Part
43	16 32. 88 35	1 3	LTEEEKNRLNFLK	15 3- 16 5	α_s 2- C N	A, B0.5-B2, C0.5, C1, D0.5, D1, E0.5	0. 18	N on	N on	Al ler ge n	Part

44	28 64. 50 14	2 3	FPQYLQYLYQGPIVLNPW DQVKR	92 -1 14	α_s 2- C N	A0.5-A2, B0.5, B1, C0.5	0. 22	N on	N on	Al ler ge n	/
45	27 08. 40 03	2 2	FPQYLQYLYQGPIVLNPW DQVK	92 -1 13	α_s 2- C N	A1-A3, B0.5-B1, C0.5, C1, D0.5, D1, E0.5	0. 14	N on	N on	Al ler ge n	/
46	90 3.5 29 0	7	NRLNFLK	15 9- 16 5	α_s 2- C N	A1, A2	0. 58	N on	-		Part
47	40 57. 07 78	3 3	ALNEINQFYQKFPQYLQY LYQGPIVLNPWDQVK	81 -1 13	α_s 2- C N	A1	0. 03	N on	N on	Al ler ge n	/
48	42 13. 17 89	3 4	ALNEINQFYQKFPQYLQY LYQGPIVLNPWDQVKR	81 -1 14	α_s 2- C N	A1	0. 07	N on	N on	Po te nti al Al ler ge n	/
49	12 50. 56 26	1 1	EQLSTSEENSK	12 6- 13 6	α_s 2- C N	B, C0.5,C2, C3,D1,D3 ,D4,E0.5- E2	0. 04	N on	N on	Al ler ge n	/
50	19 80. 85 48	1 6	FQSEEQQTDELQDK	33 -4 8	β - C N	All	0. 06	N on	N on	Al ler ge n	/
51	10 12. 51 64	8	HKEMPPFK	10 6- 11 3	β - C N	All	0. 62	N on	-		/

52	82 9.44 6	7	AVPYPQR	17 7- 18 3	β - C N	All	0. 57	N on	-		ACE inhibit or; ⁴⁰ Antiox idative ; ⁴³
53	77 9.4 90 5	7	VLPVPQK	17 0- 17 6	β - C N	All	0. 20	N on	-		Antiox idative ; ⁴³
54	87 2.4 78 9	8	VKEAMAPK	98 -1 05	β - C N	A0.5, B0.5, B1, B3, B4, C0.5-C3, D3	0. 14	N on	-		Antiox idative ; ⁴⁴
55	15 90. 92 46	1 4	VLPVPQKAVPYPQR	17 0- 18 3	β - C N	A0.5	0. 41	N on	N on	Al ler ge n	/
56	74 1.4 42 5	7	GPFPIIV	20 3- 20 9	β - C N	A, B, C2-C4, D1-D3, E	0. 81	N on	-		ACE inhibit or
57	29 08. 59 25	2 6	DMPIQAFLLYQEPVLGPV RGPFPPIV	18 4- 20 9	β - C N	A0.5, A1, A3, B0.5-B2, C0.5, D0.5, E0.5, E1	0. 33	N on	N on	N on	/
58	21 85. 16 05	1 9	DMPIQAFLLYQEPVLGPV R	18 4- 20 2	β - C N	A, B0.5, B2, C0.5-C2, D0.5, E1	0. 55	N on	N on	Al ler ge n	Anti-in flamm atory; ⁴ ⁵
59	23 51. 07 64	1 9	IEKFQSEEQQTDELQD K	30 -4 8	β - C N	A1, A3, A4, C05, C2, D0.5, E1	0. 05	N on	N on	Al ler ge n	/

60	53 15. 84 61	4 9	IHPFAQTQSLVYPFGPIP NSLPQNIPPLTQTPVVVPP FLQPEVMGVSK	49 -9 7	β - C N	A3	0. 02	N on	N on	Al ler ge n	/
61	63 58. 24 87	5 6	YPVEPFTESQSLTLTDVE NLHLPLPLLQSWMHQPH QPLPPTVMFPPQSVLSLSQ SK	11 4- 16 9	β - C N	A1, A2	0. 13	-	N on	N on	/
62	28 01. 39 30	2 5	RELEELNVPGEIVESLSSS EESITR	1- 25	β - C N	A2, C0.5-C3, E1-E3	0. 09	N on	N on	Al ler ge n	Immun omodu lating; ⁴⁶
63	11 92. 53 29	1 1	SCQAQPTTMAR	87 -9 7	κ - C N	A1-A4, B1, B2, C2, D0.5-D2, E1, E3	0. 52	N on	N on	Al ler ge n	/
64	95 4.5 17 5	8	FFSDKIAK	17 -2 4	κ - C N	A1-A4, B0.5-B3, C0.5-C2, D0.5, D1, E0.5, E1, E2	0. 51	N on	-	-	/
65	12 50. 70 23	1 0	YIPIQYVLSR	25 -3 4	κ - C N	A0.5, A1, A3, B2, B3, C1, E0.5, E1	0. 28	N on	-	-	Immun omodu lating; ⁴ ⁷ ACE inhibit or; ⁴⁸ Opioid antago nist; ⁴⁹

666	1979.084	18	SPAQILQWQVLSNTVPAK	69-86	κ -C N	A, B0.5-B3, C0.5-C2, C4, D0.5-D2, E0.5-E3	0.64	N on	N on	Al ler ge n	/
67	1607.8395	14	HPHPHLSFMAIPPK	98-111	κ -C N	A0.5, A1	0.80	N on	N on	Al ler ge n	/
68	1794.9992	16	AQILQWQVLSNTVPAK	71-86	κ -C N	A0.5	0.39	N on	N on	Al ler ge n	/
69	783.4677	7	MAIPPKK	106-112	κ -C N	A1, B0.5, B3, C0.5, C2, E0.5, E2	0.51	N on	-	-	Antithr omboti c; ⁵⁰
70	1268.6109	10	QEQNQEQPIR	1-10	κ -C N	C4, D3	0.19	N on	-	-	/

*The above peptides were obtained from the bovine casein hydrolysates with different enzyme concentration and enzymatic time, but the same concentration of substrate (4% w/v), temperature (37°C) and pH (8.0). A, B, C, D and E represent the hydrolysis with the enzyme concentration of 1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹, respectively; 0.5, 1, 2, 3 and 4 represent the hydrolysis time of 30 min, 60 min, 120 min, 180 min and 240 min, respectively; for example, “B1” represents the hydrolysis was conducted at the condition of 2000 u g⁻¹ of enzyme concentration and 60 min of hydrolysis time. “All” represents the peptides identified in all samples; “Non” represents non-allergen or non-toxicity; “-” indicates that the data are not

available using the present methods; “/” indicates that there are no bioactive report about the peptides. “Part” indicates that part of the peptide sequence was reported to be a bioactive peptide with a certain activity.

Table 2. Comparison of the number of peptides identified from α_{s1} -, α_{s2} -, β - and κ -casein under different trypsin hydrolysis conditions.

Concentration	Time	α_{s1} -casein	α_{s2} -casein	β -casein	κ -casein	Total
1000 u g ⁻¹	30 min	13	16	9	5	43
	60 min	19	17	9	8	53
	120 min	16	15	9	3	43
	180 min	15	11	9	4	39
	240 min	12	9	7	3	31
2000 u g ⁻¹	30 min	17	17	8	3	45
	60 min	13	16	7	3	39
	120 min	13	11	7	4	35
	180 min	12	10	6	4	32
	240 min	11	10	6	0	27
3000 u g ⁻¹	30 min	15	16	9	3	43
	60 min	13	13	7	3	36
	120 min	13	9	9	4	35
	180 min	11	10	7	0	28
	240 min	13	7	5	2	27
4000 u g ⁻¹	30 min	16	14	7	3	40
	60 min	14	13	6	3	36
	120 min	12	7	7	2	28
	180 min	11	10	6	1	28
	240 min	11	7	5	0	23
5000 u g ⁻¹	30 min	15	13	7	4	39
	60 min	14	9	9	4	36
	120 min	12	8	6	3	29
	180 min	11	6	6	2	25
	240 min	11	6	5	0	22

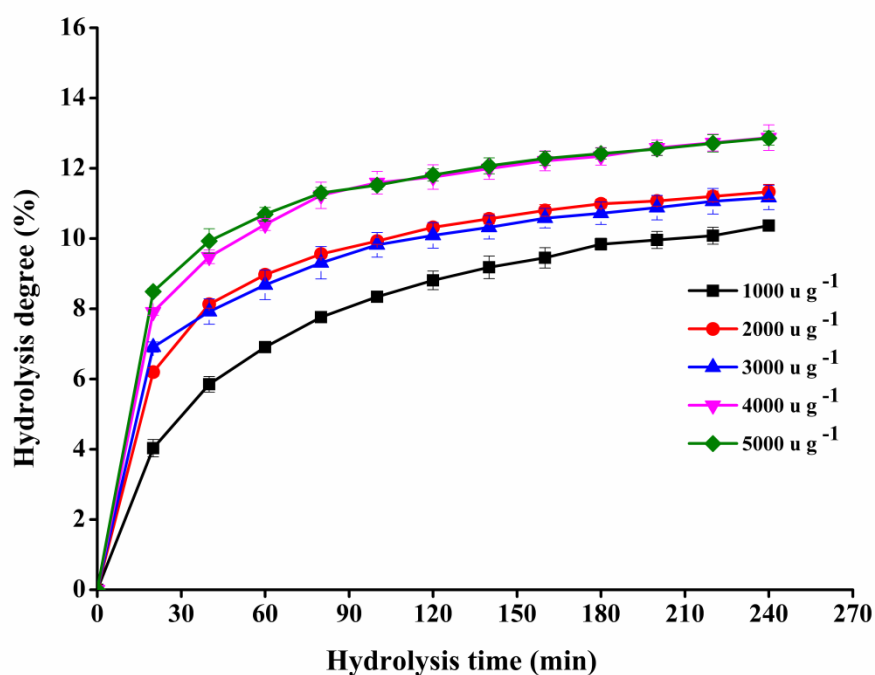


Fig. 1. Hydrolysis curves of casein treated with different enzyme concentrations. The hydrolytic reaction was carried out at 37 °C at constant pH 8.0, and with varying trypsin concentrations of 1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹ for different hydrolysis times ranging from 0 min to 240 min.

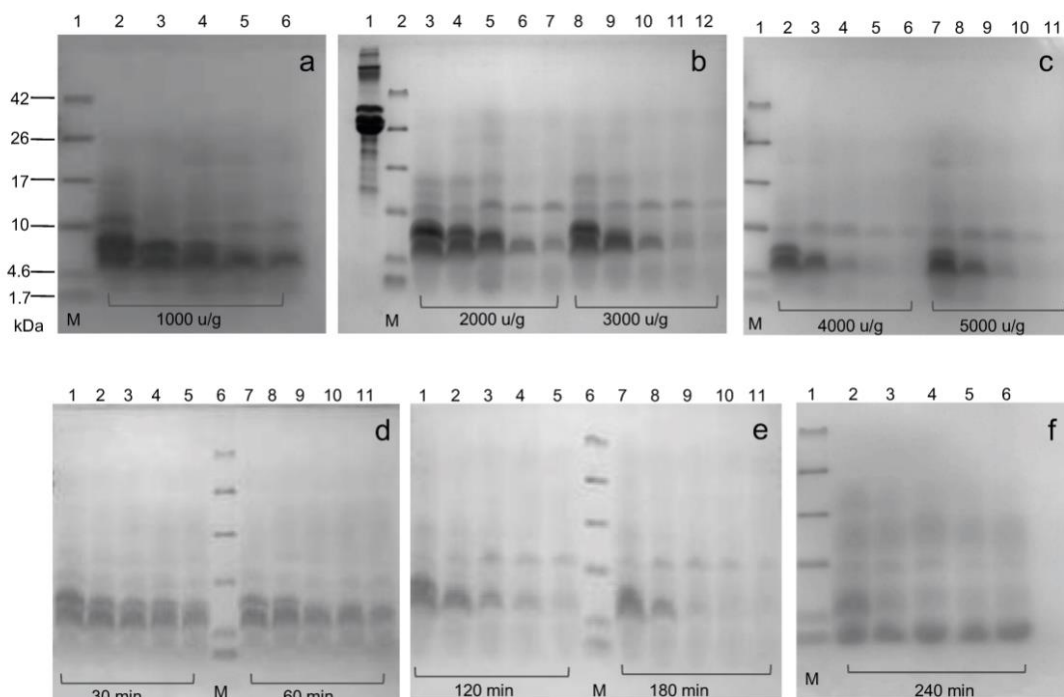


Fig. 2. Tricine-SDS-PAGE of casein hydrolysates. (a)~(c) are the profiles of peptides produced under different trypsin concentrations, (d)~(f) for different hydrolysis times. (a) H1K group. Lane 1: marker; Lane 2~6: hydrolysis for 30~240 min in the H1K group. (b) H2K and H3K groups. Lane1: natural casein; Lane 2: marker; Lane 3~7: hydrolysis for 30~240 min in the H2K group; Lane 8~12: hydrolysis for 30~240 min in H3K group. (c) H4K and H5K groups. Lane 1: marker; Lane 2~6: hydrolysis for 30~240 min in the H4K group; Lane 7~11: hydrolysis for 30~240 min in the H5K group. (d) H30 and H60 groups. Lane 1 to 5: $E/S = 1000 \sim 5000 \text{ u g}^{-1}$ in H30 group; Lane 6: marker; Lane 7 to 11: $E/S = 1000 \sim 5000 \text{ u g}^{-1}$ in the H60 group. (e) H120 and H180 groups. Lane 1 to 5: $E/S = 1000 \sim 5000 \text{ u g}^{-1}$ in H120 group; Lane 6: marker; Lane 7~11: $E/S = 1000 \sim 5000 \text{ u g}^{-1}$ in H1800 group. (f) H240 group. Lane 1: marker; Lane 2~ 6: $E/S = 1000 \sim 5000 \text{ u g}^{-1}$ in H240 group.

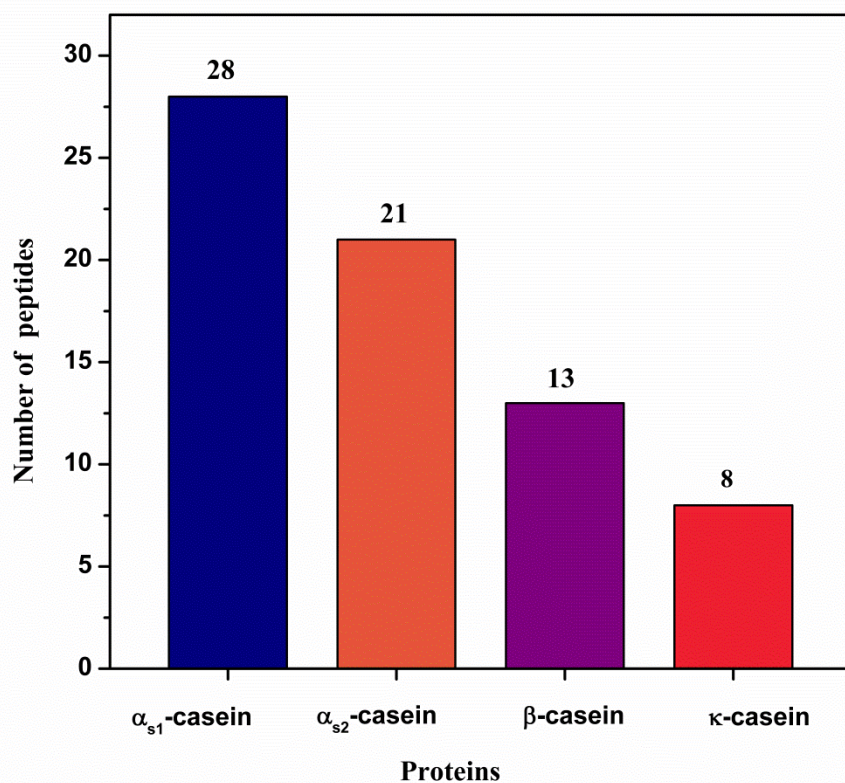


Fig. 3. Total number of peptides derived from α_{s1} -, α_{s2} -, β - and κ -casein identified from all casein hydrolysates. All casein hydrolysates represent 25 casein hydrolysates samples, which were produced by casein hydrolysis conducted for varying hydrolysis times (30 min, 60 min, 120 min, 180 min and 240 min) and trypsin concentration (1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹).

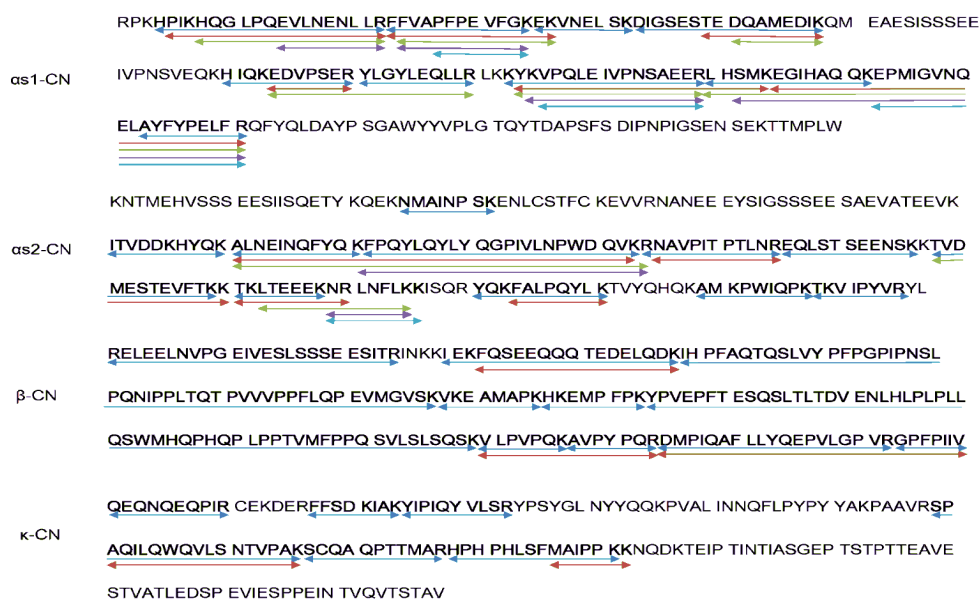


Fig. 4. Overlay of the identified peptides in casein molecules from all casein hydrolysates.

All arrows (blue, dark red, green, purple and sky blue) represent the peptides identified in all casein hydrolysates. All casein hydrolysates refer to 25 casein hydrolysates samples, which were produced by casein hydrolysis conducted with different hydrolysis time (30 min, 60 min, 120 min, 180 min and 240 min) and trypsin concentration (1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹).

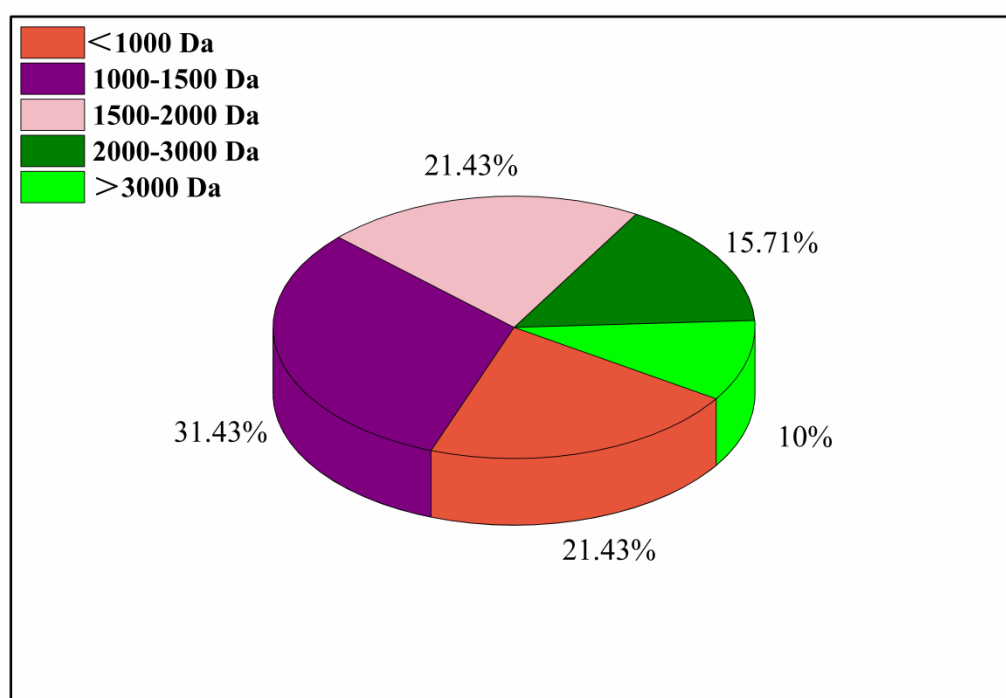


Fig. 5. Molecular weight distribution of peptides identified from all casein hydrolysates. All casein hydrolysates refer to 25 casein hydrolysates samples, which were produced by casein hydrolysis conducted with different hydrolysis time (30 min, 60 min, 120 min, 180 min and 240 min) and trypsin concentration (1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹).

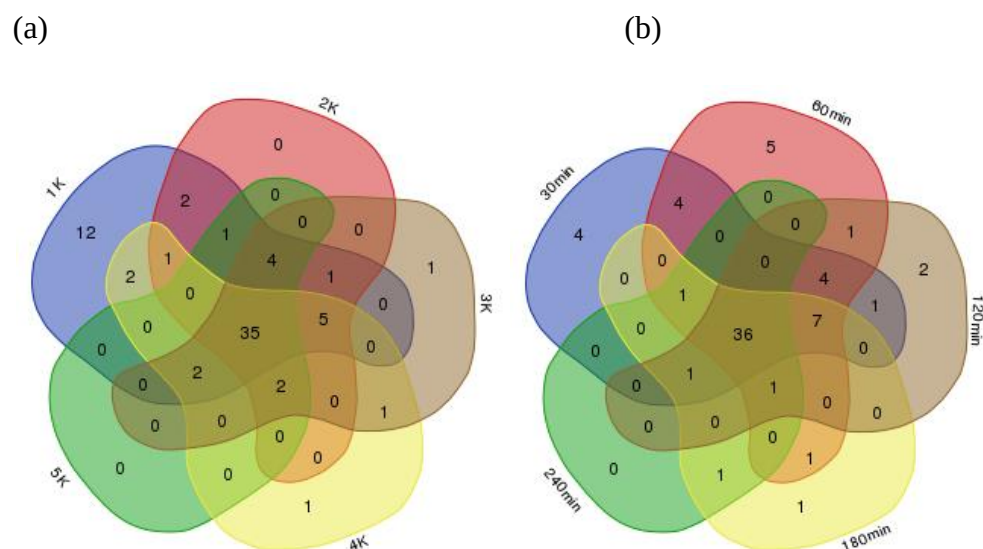


Fig. 6. Venn diagram of the peptides identified from the casein hydrolysates with (a) different amount of enzyme (1000 u g-1, 2000 u g-1, 3000 u g-1, 4000 u g-1 and 5000 u g-1) and (b) hydrolysis time (30 min, 60 min, 120 min, 180 min and 240 min).

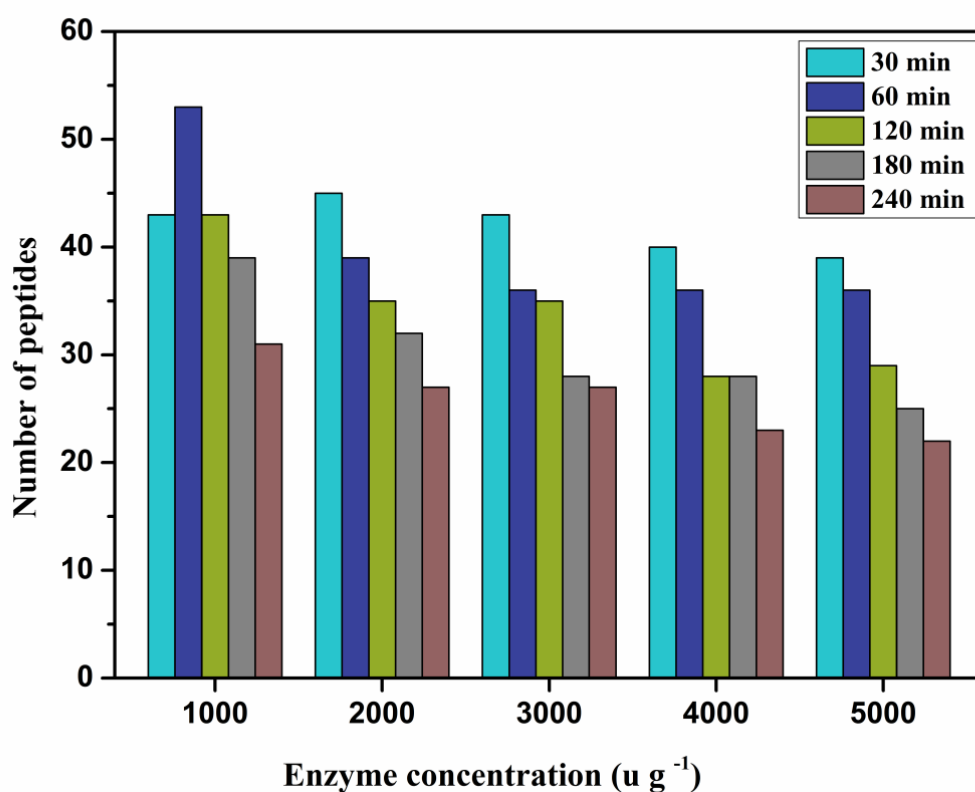


Fig. 7. Number of peptides identified under different enzymatic hydrolysis conditions. Hydrolysis time contains 30 min, 60 min, 120 min, 180 min and 240 min. Enzyme concentration includes 1000 u g⁻¹, 2000 u g⁻¹, 3000 u g⁻¹, 4000 u g⁻¹ and 5000 u g⁻¹.