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Product distribution of casein tryptic hydrolysis based on HPSEC analysis and molecular mechanism

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Abstract

Tryptic hydrolysis of whole casein was analyzed by high performance size exclusion chromatography (HPSEC) in combination with the degree of hydrolysis (DH). In terms of chromatograms obtained at different DH values and mass percentage calculated equations established by the normalization method, the complex process of enzymatic reaction and the molecular mass distribution of multiple hydrolysates were quantitatively characterized via 2-D plot. Based on the information of casein micelle structure, the possible reaction mechanism was deduced from a series of chromatographic results and experimental analysis. Taking into account the primary structure of whole casein and the target amino acid of trypsin, the distribution of theoretical peptides was accurately calculated by determining the split sites of complete enzymatic hydrolysis. According to the relationship between retention time and molecular mass, the corresponding chromatogram absorption peaks of active peptides in hydrolysates were identified, and caseinophosphopeptides (CPPs) sequence was also characterized.

Keywords: Caseinophosphopeptides; Chemical processes; Chromatography; Enzyme; Molecular biology; Peptide mapping

1. Introduction

Enzymatic hydrolysis of proteins is an important bioprocess to improve the physical, chemical, functional and nutritional properties of original proteins. Through further separation and purification, many active peptides can be isolated from the hydrolysates, which possess a lot of biological properties such as mineral binding, opioid activity, growth enhancer for bifidobacteria, anticancer activity, and regulation of the blood pressure or the immune system (Poch & Bezkorovainy, 1991; Fait, Migliore, & Jollés, 1993; Holt, Wahlgren, & Drakenburg, 1996; Kim & Chung, 1999). So these peptides will have various applications in food, drug, cosmetic, and some other fields (Kitts & Yuan, 1992).

In order to prepare target active peptides with high purity and good yield, it is necessary to study the complicated bioreaction from the viewpoint of molecular level and to characterize the multiple products in terms of their physical–chemical properties (Gonzalez, Camacho, & Jurado, 1994). All of which are helpful to establishing a reasonable relationship between structure and functionality or to elucidating the molecular mechanism of peptide activity. However, enzymatic hydrolysis is so complicated (diversity of the reaction components; variety of the reaction types; complexity of the reaction networks) that it is difficult to analyze and model the process via the conventional way.

Casein is one of the most valuable food proteins, because it is the source of many bioactive peptides such as CPPs, casoxins, casomorphins and immunopeptides (Chiba, Tani, & oshikawa, 1989; McDonagh & FitzGerald, 1998). Trypsin is a specific enzyme and its target amino acids are Arg and Lys. In order to more accurately monitor the process of hydrolysis reaction and to quantitatively calculate the molecular mass distribution of multiple products, this work intends to propose a new method that combines degree of hydrolysis (DH) concept and high performance size exclusion chromatography (HPSEC) technique for reaction characterization of casein tryptic hydrolysis. Based on the molecular biology information, the distribution of theoretical hydrolysis peptides can be calculated by determining the possible split sites. The corresponding reaction process can be deduced from a series of chromatograms. Furthermore, CPPs mapping can also be characterized from the molecular level.

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2. Materials and methods

2.1. Materials

Casein was obtained from Tianjin Hematology Institute, Chinese Academy of Medical Sciences. Its content was 86% (w/w) by protein weight using the Kjeldahl's method. Prior to use, casein was purified by precipitation and ultrafiltration.

Trypsin was purchased from LTI (GIBCO). The maximum activity of enzyme was 4 μ/mg at the optimum conditions (40°C, pH 8). It was stable from 20°C to 45°C and at pH 7–9. The enzyme can be deactivated by heating to 100°C for 5 min or adding trichloroacetic acid to pH 4.0.

The substances used as standards were listed in Table 1. High-quality purified water was distilled from the deionized water. All the other reagents were of HPLC grade.

2.2. Preparation and pretreatment of casein hydrolysate

Casein was dissolved in deionized water, then adjusted to pH 8.0 and heated up to 40°C. Trypsin was added at 1/50 (w/w) of casein concentration. Hydrolysis was carried out at constant temperature with pH maintained at 8.0 by the addition of 0.1 mol/l NaOH. The reaction was stopped at different DH values (monitored by the *pH-stat* method) via the addition of trichloroacetic acid to pH 4.0. Prior to chromatographic analysis, all hydrolysate solutions were prefiltered through a 0.45 μm millipore film and degazed with ultrasonic cleaner.

2.3. Chromatographic operating conditions

The chromatographic column used was a Waters-PROTEIN-PAK 125 (84601) which contained silica-based packing with a neutral hydrophilic moiety bound to the surface providing excellent recovery and resolution for aqueous peptide especially suited for molecular mass range 1000–80,000 Da.

The mobile phase was 0.1 mol/l phosphate solution adjusted to pH 7.0. Its flow rate and pressure were 0.5 ml/min and 4 MPa. Detection was performed by UV-light absorption at 214 nm, and the whole system was maintained at constant temperature 25°C. After the equilibration between mobile phase and packing was established, the aliquot of 20 μl samples was injected into HPSEC apparatus for analysis.

Table 1
List of substances used as internal standards

Number	Substance	Molecular mass
1	Bovine serum albumina[a]	66,409
2	Ovalbumina[a]	43,000
3	Bovine hemoglobina[a]	31,000
4	Cytochrome Cb[b]	12,327
5	Vitamin B_{12}[b]	1355

[a]Purchased from S&T company of hematology institute, Chinese academy of medical sciences.
[b]Purchased from Sigma company.

3. Theoretical aspects

3.1. Definition and measurement of DH value

DH is defined from the molecular level as the ratio of broken peptide bonds to total peptide bonds. The addition of base to maintain constant pH is proportional to the DH value and it can be expressed by the following equation (Bressollier, Petit, & Julien, 1988):

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100\% = \frac{B \times N_b \times 1000}{MP \times h_{\mathrm{tot}} \times \alpha} \times 100\%, \quad (1)$$

where B is the volume of base consumption (in l); N_B is the normality of the base (in mol/l); MP is the mass of protein ($N \times 6.25$, in g); h_{tot} is the total number of peptide bonds (in mmol/g); α is the average degree of dissociation of $\alpha - NH_2$.

3.2. Definition of polypeptide molecular mass

According to the expression of macromolecular weight in polymer chemistry, the average molecular mass of enzymatic hydrolysates are defined as follows:

$$\text{Average quantity molecular mass}: \quad M_n = \frac{\sum N_i M_i}{\sum N_i}. \quad (2)$$

$$\text{Average weight molecular mass}: \quad M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i}. \quad (3)$$

$$\text{Distribution index of molecular mass}: \quad D = \frac{M_w}{M_n}. \quad (4)$$

3.3. Derivation of functional relationship between mass percentage and peak area

In order to characterize the molecular mass distribution of hydrolysates accurately, it is necessary to revise the peak areas. Suppose that the reaction system is divided into n components and detected at m DH values. Through direct integration of absorption peaks in accordance with the range of molecular mass, the area of component i at DH j is expressed as $A_i^j = f_i M_i^j$ ($0 < i < n$, $0 < j < m$). Thus, a matrix of $n \times m$ elements is constructed for all n components and m DH values:

$$\begin{bmatrix} A_1^1 & \cdots\cdots & A_1^j & \cdots\cdots & A_1^m \\ \vdots & & \vdots & & \vdots \\ A_i^1 & \cdots\cdots & A_i^j & \cdots\cdots & A_i^m \\ \vdots & & \vdots & & \vdots \\ A_n^1 & \cdots\cdots & A_n^j & \cdots\cdots & A_n^m \end{bmatrix}, \quad (5)$$

where A is area; M is mass; the superscript is DH value; and the subscript is component.

Take component i for example, according to a quantitative correction factor method, i.e. letting

$$F_i^{(1)} = \frac{f_i}{f_1} \ldots F_i^{(i-1)} = \frac{f_i}{f_{i-1}},$$
$$F_i^{(i+1)} = \frac{f_i}{f_{i+1}} \ldots F_i^{(n)} = \frac{f_i}{f_n} \tag{6}$$

then the following relationship between mass percentage and peak area is established in terms of the normalization method:

$$\begin{aligned}
\frac{M_i^1}{M_{\text{total}}} &= \frac{A_i^1}{F_i^{(1)}A_1^1 + \cdots + F_i^{(i-1)}A_{i-1}^1 + F_i^{(i+1)}A_{i+1}^1 + \cdots + F_i^n A_n^1 + A_i^1} \\
&\vdots \qquad\qquad \vdots \\
\frac{M_i^j}{M_{\text{total}}} &= \frac{A_i^j}{F_i^{(1)}A_1^j + \cdots + F_i^{(i-1)}A_{i-1}^j + F_i^{(i+1)}A_{i+1}^j + \cdots + F_i^n A_n^j + A_i^j} \\
&\vdots \qquad\qquad \vdots \\
\frac{M_i^m}{M_{\text{total}}} &= \frac{A_i^m}{F_i^{(1)}A_1^m + \cdots + F_i^{(i-1)}A_{i-1}^m + F_i^{(i+1)}A_{i+1}^m + \cdots + F_i^n A_n^m + A_i^m},
\end{aligned} \tag{7}$$

where F is correction factor.

Furthermore, the following set of equations is deduced from above functional relationship:

$$\begin{aligned}
&F_i^{(1)}(A_1^2 - A_1^1) + \cdots + F_i^{(i-1)}(A_{i-1}^2 - A_{i-1}^1) + F_i^{(i+1)}(A_{i+1}^2 - A_{i+1}^1) + \cdots + F_i^n(A_n^2 - A_n^1) + (A_i^2 - A_i^1) = 0 \\
&\qquad\vdots \qquad\qquad\qquad\vdots \qquad\qquad\qquad\vdots \qquad\qquad\qquad\vdots \\
&F_i^{(1)}(A_1^j - A_1^1) + \cdots + F_i^{(i-1)}(A_{i-1}^j - A_{i-1}^1) + F_i^{(i+1)}(A_{i+1}^j - A_{i+1}^1) + \cdots + F_i^n(A_n^j - A_n^1) + (A_i^j - A_i^1) = 0 \\
&\qquad\vdots \qquad\qquad\qquad\vdots \qquad\qquad\qquad\vdots \qquad\qquad\qquad\vdots \\
&F_i^{(1)}(A_1^m - A_1^1) + \cdots + F_i^{(i-1)}(A_{i-1}^m - A_{i-1}^1) + F_i^{(i+1)}(A_{i+1}^m - A_{i+1}^1) + \cdots + F_i^n(A_n^m - A_n^1) + (A_i^m - A_i^1) = 0
\end{aligned} \tag{8}$$

by solving the linear set of Eqs. (8) and substituting the unknowns $F_i^1, \ldots, F_i^{(i-1)}, F_i^{(i+1)}, \ldots, F_i^n$ into Eqs. (7), the mass percentage of component i at m DH values is calculated. In a similar way, other components mass percentages are also determined.

3.4. Calculation of theoretical hydrolysis peptides distribution

The program is composed of two modules. One performs the calculation of peptide molecular mass; the other carries out the prediction of hydrolysates distribution. Hereon assuming that proteases only attack the target peptide bonds according to their specificity and every possible split site of protein are cleaved at the final stage of the hydrolysis.

4. Results and discussion

4.1. Standard curve of molecular mass

By the regression analysis of five standard proteins' chromatogram (Fig. 1), the good linear relationship of molecular mass and retention time is determined as

$$\log MW = -0.12t_r + 6.59. \tag{9}$$

4.2. Chromatographic analysis of whole casein

Fig. 2a is the chromatogram of original substrate before hydrolysis. Due to high purity, the main component of casein corresponds to a single dominant peak ($t_r = 12.55$ min), and its molecular mass approximately equals to 121 kDa, which is much greater than that of four monomers, i.e. α_{s1}-, α_{s2}-, β-, and κ-casein.

The above phenomenon indicates that the whole casein much more likely exists in *Waugh* micelles. This general accepted structure has been described as a core-coat model, because it is composed of a hydrophobic association core of α_{s1}-, α_{s2}-, and β-casein and a monolayer protective coat of $\kappa-$ casein.

4.3. Reaction process of casein tryptic hydrolysis

4.3.1. Chromatogram analysis

Fig. 2b–d are the chromatograms of enzymatic hydrolysates at DH = 5%, 10%, and 15%, corresponding to the

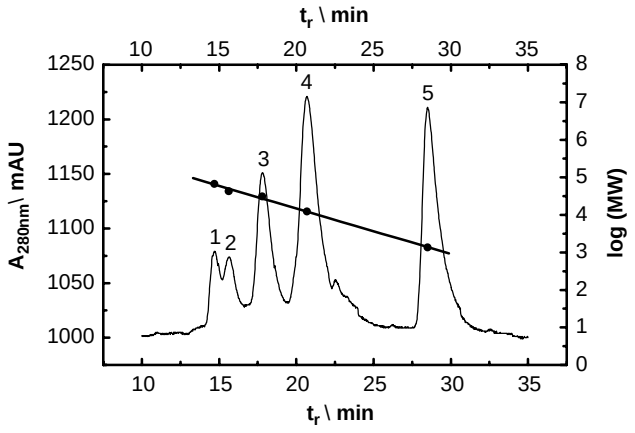

Fig. 1. Chromatogram of standard proteins: (1) bovine serum albumin; (2) ovalbumin; (3) bovine hemoglobin; (4) cytochrome C; and (5) vitamin B_{12}.

hydrolysis times 9, 31, and 90 min. The retention time of main absorption peaks and corresponding molecular mass determined by Eq. (9) are shown in Table 2.

In contrast to Fig. 2a, the single peak of original substrate rapidly disappears and it is evidently divided into several quite strong absorption peaks, which correspond to different molecular mass during $t_r = 17$–32 min. Moreover, these peaks area change continuously as reaction proceeds. The above phenomenon suggests that *Waugh* micelles are easily depolymerizated by means of enzymatic hydrolysis, and simultaneously all kinds of monomers are released. Furthermore, due to the specificity of trypsin, the peptide chains of monomers are cleaved into relatively concentrative fragments including macropeptides (15.7–3.7 kDa, peaks 3 and 4); polypeptides (3.7–0.8 kDa, peaks 5 and 6); and oligopeptides (< 0.8 kDa, peak 7).

There are some active peptides in the above hydrolysis fragments, which are generally called CPPs. As the name implies, these peptides contain a common motif, i.e. a sequence of *SerP-SerP-SerP-Glu-Glu*. Considering their highly polar residues and their close active domains, it is usually difficult to further hydrolysis as soon as they form.

4.3.2. Molecular mass distribution of whole casein tryptic hydrolysis

As shown in Fig. 2, the total absorption peak areas at different DH values increase with the process of hydrolysis reaction. Since peak area should be proportional to substance content, the phenomenon seems to contrary to the law of mass conservation. However, this is just the key to quantitative characterization of multiple hydrolysates. To be more precise, the proportional relationship is only valid for a certain absorption peak area and its corresponding hydrolysates. In other words, different peptide fragments have different correction factor F. So the total peak areas will change with the variation of hydrolysates composition, and area values of each component must be revised for the accurate characterization. The higher degree of hydrolysis, the bigger peak area, the less content of long peptide chains, and the more content of middle or short peptide chains, all of which demonstrate that oligopeptides have stronger absorption than macropeptides at the same detection wavelength and mass concentration. Such experimental results can be elucidated in terms of molecular structure, i.e. most accessible residues are buried into the compact original substrate; while enzymatic hydrolysis makes the target peptide bonds that may cause intensive absorption expose to the surface of generated peptide fragments.

Fig. 3 is 2-D plot of mass percentage vs. DH value or molecular mass. According to the normalization method, peak areas determined from integration of chromatographic curves are revised to calculate the mass percentage, and the plot is obtained by solving a set of linear equations. The detailed method has been derived in Section 3.3.

From the viewpoint of enzymatic hydrolysis process (Fig. 3a), it can be seen that: (1) original substrate disappears rapidly at the initial stage of reaction ($DH < 5\%$),

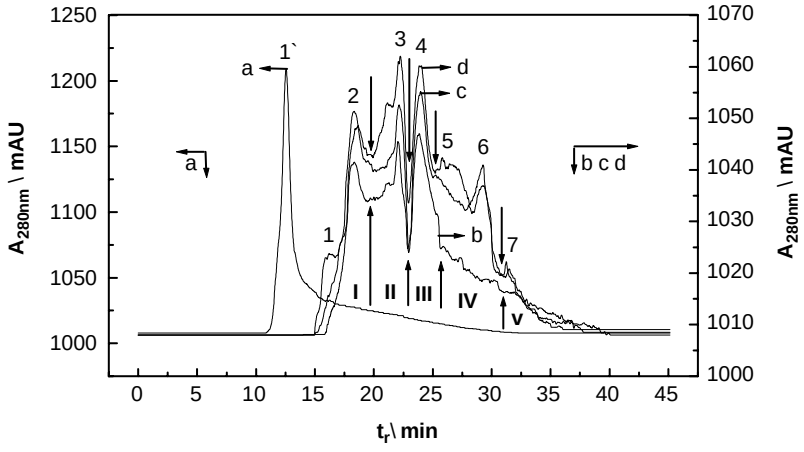

Fig. 2. Chromatograms of casein tryptic hydrolysis: (a) whole casein; (b) $DH = 5\%$; (c) $DH = 10\%$; and (d) $DH = 15\%$.

Table 2
The relationship of retention time, molecular mass and peptide fragments for tryptic hydrolysis of casein

Experimental results			Theoretical analysis	
Peak	t_r (min)	MW (10^3 Da)	Functional peptides (containing $SerP$)	Common peptides
a1′	12.55	121.3	Whole casein	
b1	14.02–16.20–16.72	80.8–44.3–38.3	Casein core (be composed of α_{s1}-, α_{s2}-, and β-casein)	
b2 \ c2 \ d2	16.72–18.33–19.95	38.3–24.6–15.7	Monomer: α_{s1}-, α_{s2}-, β- and κ-casein	
b3 \ c3 \ d3	19.95–22.15–22.95	15.7–8.6–6.9	γ-casein and other combination peptides	
b4 \ c4 \ d4	22.95–23.98–25.20	6.9–5.2–3.7	α_{s1}-7P (43–79)[a]\κ-1P(117–169)	α_{s1}(152–193) \ β (49–97) \ β (114–169)
d5	25.20–25.80–28.15	3.7–3.1–1.6	α_{s1}-2P (43–58) \ α_{s1}-5P (59–79) \α_{s1}-1P (106–119) \ α_{s2}-4P (1–21) \ α_{s2}-4P (46–70)\ β-4P (1–25)[b] \ β-1P (33–48)	α_{s1} (8–22) \ α_{s1} (23–34) \ α_{s1} (133–151) \ α_{s2} (92–113) \ β (177–183) \ β (184–202) \ κ (1–10) \ κ (47–63) \ κ (69–86)
d6	28.15–29.25–30.80	1.6–1.2–0.8	α_{s2}-1P (138–149) \ α_{s2}-2P (126–136)	α_{s1} (84–90) \ α_{s1} (91–100) \ α_{s1} (125–132) \ α_{s2} (25–32) \ α_{s2} (33–41) \ α_{s2} (81–91) \ α_{s2} (115–125) \ α_{s2} (174–181) \ α_{s2} (182–188) \ β (170–176) \ κ (25–34) \ κ (35–46) \ κ (87–97) \ κ (98–111)
d7	30.80–31.23–32.52	0.8–0.7–0.5	α_{s1}-1P (37–42)	α_{s1} (4–7) \ α_{s1} (80–83) \ α_{s1} (120–124) \ α_{s1} (194–199) \ α_{s2} (42–45) \ α_{s2} (71–76) \ α_{s2} (77–80) \ α_{s2} (153–158)\ α_{s2} (161–165) \ α_{s2} (167–170) \ α_{s2} (192–197) \ α_{s2} (200–205)\ β (100–105) \ β (108–113) \ β (203–209) \ κ (17–21) \ κ (64–68)\ κ (113–116)

[a] α_{s1}-7P (43–79) is α-CPP ($MW = 4600$).
[b] β-4P (1–25) is β-CPP ($MW = 3100$).

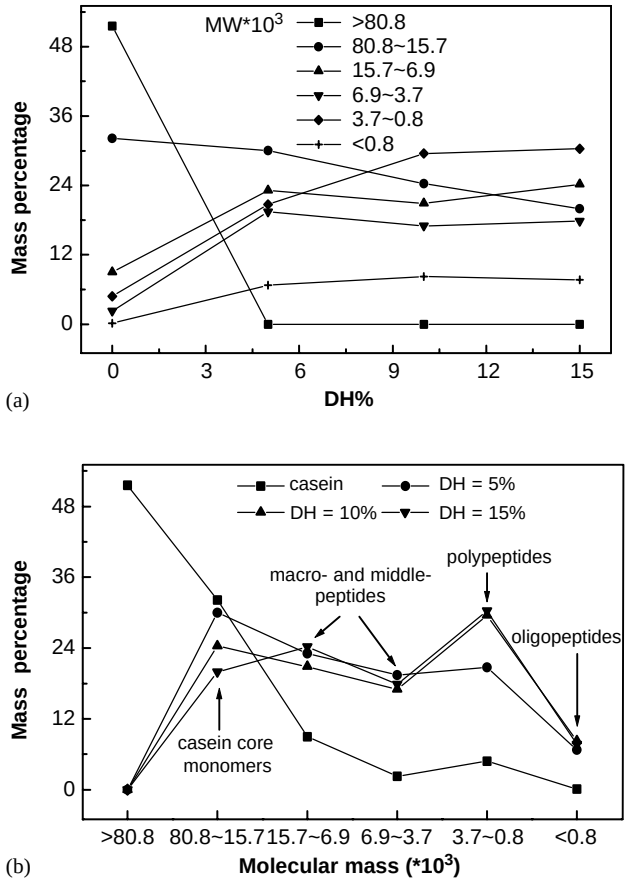

Fig. 3. Molecular mass distribution of whole casein tryptic hydrolysis: (a) process of enzymatic hydrolysis; and (b) variation in the composition of hydrolysates.

and some oligopeptides generate simultaneously, which is about 7%; (2) as reaction proceeds (from $DH = 5\%$ to 15%), the content of macromolecule including casein core and monomers casein (80.8–15.7 kDa) is declining, while the content of micromolecule such as polypeptides (3.7–0.8 kDa) is rising; (3) the different primary and space structure of each peptide family leads to the different variation rate in mass percentage, but due to a decrease in the concentration of sensitive peptide bonds and an inactivation of trypsin caused by temperature, pH or autolysis, the variation rate of component content slows down gradually; (4) on account of the specific and complete hydrolysis of trypsin, the percentage of each peptide family tends towards constant value at final stage.

From the viewpoint of variation in the hydrolysates composition (Fig. 3b), it can be seen that: (1) casein core and monomers casein (80.8–15.7 kDa) show a continuous decrease in percentage, which means they are easily depolymerizated and degraded; (2) the content of middle peptide chains (including 15.7–6.9 and 6.9–3.7 kDa) appears as fluctuating, which suggests there are a complex reaction network and a strong interrestriction among these kinds of hydolysates, so the total apparent degradation rate of them

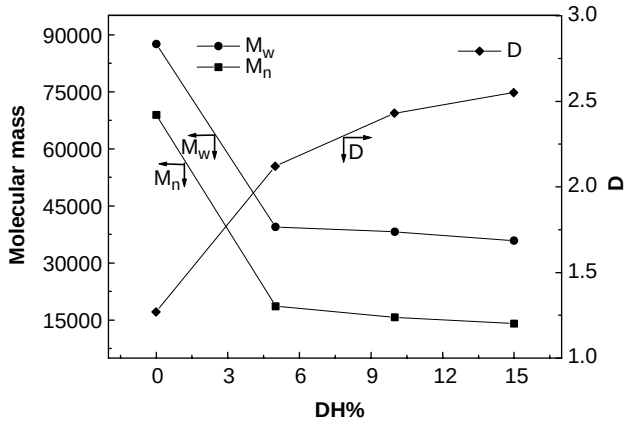

Fig. 4. Average molecular mass and distribution index of casein–trypsin hydrolysis system.

approximately equals the total formation rate; (3) polypeptides (3.7–0.8 kDa) present a very pronounced increase in percentage from 5% (time = 0 min) to 30% (time = 90 min), and they have the highest content value at final stage, which indicates that this part of hydrolysates are easy to be generated, but difficult to be degraded, so they are much more likely the end products of the reaction; (4) oligopeptides (< 0.8 kDa) quickly form at initial stage ($DH = 5\%$), then slowly increase (only from 7% to 8%), and they have the lowest mass percentage at the end of the reaction, which is probably due to the following factors: the open structure of monomers casein are easily attacked by trypsin, and the great specificity of trypsin enables the number of oligopeptide fragments to be constant at the high degree of hydrolysis.

As shown in Fig. 4, during the hydrolysis, the average molecular mass continuously decreases, but the distribution index gradually increases. This result further bears out that tryptic hydrolysis can cause the macro-molecular substrate to be degraded into multiple and divergent peptide fragments step by step.

In addition, the above experimental results and research method can also be used to effectively prepare active peptides. For example, it is reported that the molecular mass of main components of CPPs are 4600 and 3100, respectively (Hirayama, Toyota, & Yamaguchi, 1992), which correspond to peaks 4 and 5 of experimental chromatogram. According to the mass percentage of those peptides obtained from 2-D plot, the reasonable hydrolysis conditions can be roughly determined, i.e. it is necessary to react at least 90 min for casein–trypsin system to prepare CPPs at pH = 8 and 40°C.

4.4. Analysis of split sites and deduction of reaction mechanism

By querying protein databank it is known that (1) monomers casein are incompact and open, and their

Table 3
Information of casein structure

Casein	α_{s1}-	α_{s2}-	β-	κ-	Average
Percentage	44.7	10.5	32.9	11.9	—
MW ($\times 10^3$)	23.6	25.2	24.0	19.0	23.4
Residues	199	207	209	169	200
n[a]	21	30	15	14	19
N[b]	253	496	136	120	224

[a] n is the number of Lys and Arg residues.
[b] $N = 2n + C_n^2$, N is the number of theoretical hydrolysis peptides.

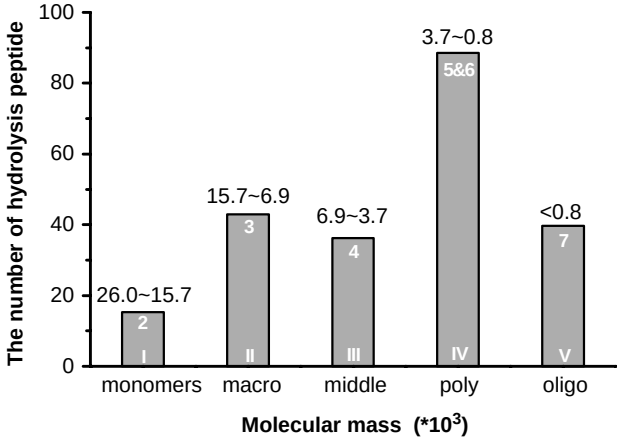

Fig. 5. The number of theoretical hydrolysis peptides vs. molecular mass distribution.

structure information is listed in Table 3; (2) trypsin is of high specificity, and it only cleaves Arg and Lys residues at the carbonyl end; and (3) complete hydrolysis enables all target peptide bonds to be attacked. Based on the above knowledge, the number of theoretical fragments is determined as 224. Their molecular mass distribution (Fig. 5) is also accurately calculated with the computer program.

Comparing the reported peptide sequence (Adamson & Reynolds, 1995, 1996) and the theoretically calculated results with the absorption peaks, the active peptides in full hydrolysates are identified (Table 2). In fact, a class of fragments with close molecular mass matches to a single peak, so that the number of fragments in hydrolysates is much more than that of absorption peaks on chromatogram. In order to establish the one-to-one relationship between fragments and peaks, more advanced analytical technology is needed such as HPLC/MS and HPLC/CEZ (Adamson & Reynolds, 1996).

As shown in Fig. 5 and Table 2, the amount of theoretical hydrolysis peptides that match to each specified absorption peak is different in evidence. For example, the number of polypeptides (3.7–0.8 kDa) is much more than the number of monomers (26–15.7 kDa), macropeptides (15.7–6.9 kDa) or oligopeprides (< 0.8 kDa). This phenomenon coincides with the experimental results (Fig. 2). From these graphs and a series of molecular structure information, the possible reaction mechanism can be deduced, which consists of three steps. (1) κ-Casein is split at Lys 116–117, then a few of oligopeptides generate; (2) *Waugh* micelle is destroyed, then α- and β-casein are released from protein core; and (3) multiple fragments with different molecular mass including maco-, poly-, and oligo-peptides come into being in succession by the specific function of trypsin. Furthermore, CPPs mapping (Table 4) that contains the different number of *SerP* residues are characterized in terms of SWISS-PORT databank, and the molecular structures of CPP's main component including α_{s1}-7P (43–79) and β-4P (1–25) are also represented as 3-D model (Fig. 6) via a molecular graphics program. Due to their compact and close structure, these functional polypeptides are difficult to be further cleaved, and are most likely the end products. Their active sites, i.e. the sequence of *SerP-SerP-SerP-Glu-Glu*, are also integrally preserved. Therefore, tryptic hydrolysis of casein is an ideal reaction system to prepare biological peptides of milk source.

5. Conclusions

(1) The combination of HPSEC technique with DH concept is a powerful method to study the protein enzymatic hydrolysis. It is not only because this new method can be widely used to follow up the complex process of reaction at any moment, but also because it can be effectively applied to quantitatively characterize the molecular mass distribution of multiple hydrolysates.

(2) Based on the molecular structural knowledge and chromatographic analysis, much useful information of casein–tryptic hydrolysis to prepare active peptides can be obtained simply and rapidly. Those include characterization of the reaction process or the composition variation via 2-D plots, prediction of the potential split site, calculation of the theoretical fragment distribution, deduction of the possible reaction mechanism, and determination of the CPPs mapping.

Table 4
CPPs mapping and corresponding HPSEC absorption peaks

Peptide name	Peptide mapping	*MM*	Peak
α_{s1}-1P (37–42)	Val-Asn-Glu-Leu-*SerP*-Lys	768	7
α_{s1}-1P (43–79)	Asp-Ile-Gly-*SerP*-Glu-*SerP*-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-Glu-Ala-Glu-*SerP*-Il3-*SerP*-*SerP*-*Serp*-Glu-Glu-Ile-Val-Pro-Asn-*Serp*-Val-Glu-Gln-Lys	4645	4
α_{s1}-1P (106–119)	Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-*SerP*-Ala-Glu-Glu-Arg	1659	5
α_{s2}-4P (1–21)	Lys-Asn-Thr-Met-Glu-His-Val-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-Lys	2745	5
α_{s2}-4P (46–70)	Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-*SerP*-*SerP*-*SerP*-Glu-Glu-*SerP*-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys	2898	5
α_{s2}-2P (126–136)	Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys	1410	6
α_{s2}-1P (138–149)	Thr-Val-Asp-Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys	1465	6
β-4P (1–25)	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-*SerP*-Leu-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Thr-Arg	3120	5
β-1P (33–48)	Phe-Gln-*SerP*-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys	2060	5
κ-1P (117–169)	Thr-Glu-Ile-Pro-Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-*Thr*-Ser-*Thr*-Pro-*Thr*-*Thr*-Glu-Ala-Val-Glu-Ser-Thr-Val-Ala-Thr-Leu-Glu-Asp-*SerP*-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val	5610	4

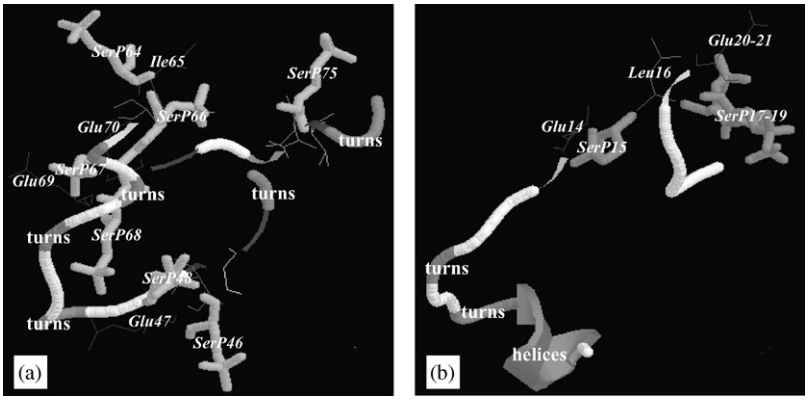

Fig. 6. Molecular structure of CPP's main components: (a) α_{s1}-7P (43–79); and (b) β-4P (1–25).

Notation

A_i^j	peak area of component i at $DH = j$
B	volume of bass consumption, L
D	distribution index of molecular mass, dimensionless
DH	degree of hydrolysis
F	correction factor, dimensionless
h_{tot}	total number of peptide bonds, mmol/g
M_I	molecular mass of component i
M_i^j	mass of component i at $DH = j$
M_n	average quantity molecular mass, Da
MP	mass of protein, g
MW	relative molecular mass, Da
M_w	average weight molecular mass, Da
n	the number of Lys and Arg residues, dimensionless
N	the number of theoretical hydrolysis peptides, dimensionless
N_b	normality of the base, mol/l
N_i	mole fraction of component i
t_r	retention time, min
α	average degree of dissociation of $\alpha - NH_2$ groups, dimensionless

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References

Adamson, N. J., & Reynolds, E. C. (1995). Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnology and Bioengineering*, *45*, 196–204.

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, *19*, 202–207.

Bressollier, P., Petit, J., & Julien, R. (1988). Enzymatic hydrolysis of plasma proteins in a CSTR ultrafiltration reactor: Performances and modeling. *Biotechnology and Bioengineering*, *31*, 650–658.

Chiba, H., Tani, F., & Yoshikawa, M. (1989). Opioid antagonist peptides derived from κ-casein. *Journal of Dairy Research*, *56*, 363–366.

Fait, A. M., Migliore, S. D., & Jollés, P. (1993). Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *Journal of Dairy Science*, *76*, 301–310.

Gonzalez, P., Camacho, F., & Jurado, E. (1994). Enzymatic hydrolysis of whey proteins: II. Molecular weight range. *Biotechnology and Bioengineering*, *44*, 529–532.

Hirayama, M., Toyota, K., & Yamaguchi, G. (1992). HPLC analysis of commercial casein phosphopeptides (CPP). *Bioscience, Biotechnology and Biochemistry*, *56*, 1126–1127.

Holt, C., Wahlgren, N. M., & Drakenburg, T. (1996). Ability of a β-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochemical Journal*, *314*, 1035–1039.

Kim, Y., & Chung, B. (1999). A novel angiotensin-I-converting enzyme inhibitory peptide from human α_{s1}-casein. *Biotechnology Letters*, *21*, 575–578.

Kitts, D. D., & Yuan, Y. V. (1992). Caseinophosphopeptides and calcium bioavailability. *Trends in Food Science and Technology*, *3*, 31–35.

McDonagh, D., & FitzGerald, R. J. (1998). Production of caseinophosphopeptides (CPPs) from sodium caseinate using a range of protease preparations. *International Dairy Journal*, *8*, 39–45.

Poch, M., & Bezkorovainy, A. (1991). Bovine milk κ-casein trypsin digest is a growth enhancer for the genus Bifidobacienum. *Journal of Agriculture and Food Chemistry*, *39*, 73–77.