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Pancreatic hydrolysis of bovine casein: Changes in the aggregate size and molecular weight distribution

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Abstract

The effect of pancreatic hydrolysis on the aggregate size and molecular weight distribution (MWD) of bovine casein was investigated by size exclusion chromatography (SEC), coupled with multiangle laser light scattering (MALLS) and dynamic light scattering (DLS). The concentrations of both casein aggregates and monomers decreased as the reaction proceeded and almost disappeared after a 10 min hydrolysis. The ratio of aggregates to monomers was maintained at 1:(2–2.5) during the initial 10 min hydrolysis, which shows that the caseins in the solution are in a dynamic equilibrium system, between aggregates and monomers. Upon limited hydrolysis, casein aggregates displayed a continuous growth in their sizes and molecular weights, together with a decrease in the intermolecular repulsion. This result was further supported by on-line DLS measurements, which showed the increase in the hydrodynamic radii and the decrease in light scattering intensities of casein hydrolysates. Moreover, the release kinetics of peptide fractions with different molecular weights were also followed. It was suggested that the increase in the hydrophobic attraction and the reduction in the protein repulsion might promote casein aggregation, during the limited pancreatic hydrolysis.

Keywords: Casein; Pancreatin; SEC–MALLS; Dynamic light scattering; Enzymatic hydrolysis

1. Introduction

Mammalian milk contains an array of nutrients such as proteins, lipids and minerals, which will deliver various bioactivities *in vivo* for the newborns. As the predominant protein in milk, casein exists in a micelle form assembled by four phosphorylated proteins, i.e. α_{S1}-, α_{S2}-, β- and κ-caseins. To date, casein has been described as a powerful source of bioactive peptides with a lot of functions, such as antihypertensive (Lopez-Fandino, Otte, & van Camp, 2006), opioid (Meisel & FitzGerald, 2000), antioxidative (Pihlanto, 2006), immunomodulating (Gill, Doull, Rutherfurd, & Cross, 2000), antimicrobial (Exposito & Recio, 2006), anticariogenic (Andrews et al., 2006) and mineral binding (Meisel & FitzGerald, 2003).

Such bioactive peptides are latent, in an inactive state, within the sequence of the precursor proteins and can be released and liberated by protease during food processing or gastrointestinal digestion. It should be noted that the various functions of bioactive peptides depend strongly on their molecular lengths. For example, the antimicrobial peptides should contain about 20–46 amino acid residues because the adequate molecular length is required to cross the membrane barrier, under certain circumstances (Nicolas & Mor, 1995). Therefore, most antimicrobial peptides are formed initially during enzymatic digestion. Antihypertensive peptides inhibit angiotensin-I-converting enzyme (ACE) and thus play a key role in blood pressure regulation. They usually contain 2–12 amino acid residues, though some of them have been identified with up to 27 residues (Lopez-Fandino et al., 2006). After the extensive hydrolysis, protein hydrolysates should be rich in low molecular weight peptides. Such small peptides, especially di- and tri-peptides, have a high intestinal absorption rate

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because they are subject to transmembrane transport (Daniel, 2004). Therefore, it is fundamental to characterise protein hydrolysates on the basis of their sizes and molecular weights for research, application and production of the bioactive peptides.

Casein micelle, the radius of which is about 25–125 nm, is a roughly spherical particle held together by colloidal calcium phosphate (Mellema, Heesakkers, van Opheusden, & van Vliet, 2000). To date, the micellisation behaviour of the individual casein components, in aqueous solution, has been well investigated (Alaimo, Wickham, & Farrell, 1999; Portnaya et al., 2006). Caseins are very prone to association due to their special hydrophobicity and charge distribution. Upon removal of calcium phosphate from casein micelles, the individual casein molecules interact with each other and form associate structures, which are called aggregates in this work (Farrell, Malin, Brown, & Qi, 2006).

It is now accepted that the biological functions of casein aggregates depend on their molecular structures and sizes. To date, the effects of pH, temperature, ionic strength and concentration on the aggregate sizes and molecular weights of sodium caseinates (without calcium phosphate) have been typically investigated (Chu, Zhou, Wu, & Farrell, 1995; Dickinson, Radford, & Golding, 2003; Lucey, Srinivasan, Singh, & Munro, 2000). Recently, it has been shown that the aggregation of casein micelles (with calcium phosphate) could also be promoted by the added plasmin (Crudden, Afoufa-Bastien, Fox, Brisson, & Kelly, 2005). Aggregation and gelation of whey proteins, another important type of milk protein, has also been detected and assessed after enzymatic hydrolysis by immobilised trypsin (Chen, Swaisgood, & Foegeding, 1994), a protease from *Bacillus licheniformis* (Otte, Lomholt, Halkier, & Qvist, 2000; Otte et al., 1997) and alcalase (Doucet, Gauthier, Otter, & Foegeding, 2003; Doucet, Otter, Gauthier, & Foegeding, 2003). In these studies, it was believed that protein aggregates and gels were formed by the changes in the intermolecular attractive forces, such as hydrophobic interaction.

One of the best current techniques for determining the size and molecular weight distribution (MWD) of protein aggregates is size exclusion chromatography–multiangle laser light scattering (SEC–MALLS) (Ye, 2006). The SEC–MALLS technique can directly provide accurate and informative data regarding the size and molecular weight of larger molecules, such as casein aggregates (Wyatt, 1993). Moreover, it can also be applied to calculate the relative MWD of the resulting peptides from enzymatic hydrolysis of casein, after the calibration of the SEC columns.

In our previous study, the release kinetics of peptides were examined by using HPLC–ESI-MS/MS during pancreatic hydrolysis of bovine casein (Su, Qi, He, Yuan, & Zhang, 2007). The aim of this work was to (1) determine the aggregate size and MWD of bovine casein by SEC–MALLS, (2) follow the pancreatin-induced changes in micelle size of bovine casein by SEC–MALLS and dynamic light scattering (DLS) and (3) analyse the MWD of the resulting peptides from pancreatic hydrolysis of bovine casein.

2. Materials and methods

2.1. Materials

Bovine casein (Product Code C7078), of technical grade, and porcine pancreatin (P7545) were obtained from Sigma Co (USA). HPLC grade Acetonitrile (ACN) was purchased from Merck Co. (Darmstadt, Germany). Decahydronaphthalene (decalin, as an index-matching liquid to reduce flare in dynamic light scattering) was purchased from Fisher Scientific (Pittsburgh, PA). Ultrapure water was obtained from an ELGA water purification unit (ELGA Ltd., Bucks, England). All other common reagents and solvents were of analytical grade obtained from commercial sources.

2.2. Pancreatic hydrolysis of bovine casein

Bovine casein, at 10 mg/ml, was digested with pancreatin (0.1 mg/ml) at 37 °C in a batch stirred tank reactor. The pH was kept at a stable value of 8.0 by adding 0.1 M NaOH solution using a pH-stat method. During the reaction, aliquots of bovine casein and pancreatin mixture were taken out at 0 (control), 2.5, 5, 10, 30, 60 and 120 min. Each aliquot was heated using boiled water for 10 min, to inactive the pancreatin and stop the reaction. The samples were lyophilised and then stored at −20 °C. The degree of hydrolysis (DH), i.e. the percentage of peptide bond cleaved during the enzymatic reaction was measured using the pH-stat method, as previously described (Su et al., 2007).

2.3. Size exclusion chromatography coupled with multiangle laser light scattering

The high-performance size exclusion chromatographic (HPSEC) system consisted of an Agilent 1100 isocratic pump (Agilent Technologies, Waldbronn, Germany), a Rheodyne 7725i injector loop of 100 μl and three columns in series, including PL aquagel-OH Guard, PL aquagel-OH MIX and PL aquagel-OH 83 (Polymer Laboratories, Shropshire, UK). The columns were thermostated at 35 °C and eluted with a mixture of water–ACN (70:30, v/v). The flow rate was 0.5 ml/min. The elution profiles were monitored by an ultraviolet (UV) detector, a BI-MwA MALLS detector equipped with a 30 mW He–Ne linearly polarised laser at a wavelength of 660 nm and a BI-DNDC differential refractometer (RI) (Brookhaven Instruments, Holtsville, NY).

The freeze-dried casein (control) and its pancreatic hydrolysates were re-dissolved in the elutant buffer at 5.0 mg/ml. All the samples were filtered through 0.45-μm filters (PALL Corp.) before injection. Bovine serum albu-

min (BSA) was used for normalising the signals of UV and RI detectors relative to those of MALLS detector. The WinGPC 7.2 software (Polymer Standard Service, Mainz, Germany) was applied to collect and process the elution data.

The basic light scattering equation (Meyer & Morgenstern, 2003) is

$$\frac{KC}{R(\theta)} = \frac{1}{M_w}\left[1 + \frac{16\pi^2 n_s^2}{3\lambda^2}\langle R_g^2\rangle \sin^2\left(\frac{\theta}{2}\right)\right] + 2A_2C, \quad (1)$$

where $R(\theta)$ is the excess intensity of scattered light at the angle θ, K is the light scattering optical constant, C is the protein concentration, M_w is the weight-average molecular weight, λ is the wavelength of laser light, n_s is the refractive index of the solvent, $\langle R_g^2\rangle$ is the mean square radius of the molecules, A_2 is the second virial coefficient. The optical constant K depends on the scattering properties of the solution, i.e.

$$K = \frac{4\pi^2 n_s^2 (dn/dc)^2}{N_A \lambda^4}, \quad (2)$$

where N_A is Avogadro's number, dn/dc is the refractive index increment of protein solution as a function of concentration. A dn/dc value of 0.186 ml/g was applied for the molecular weight calculations (Wen, Arakawa, & Philo, 1996).

During the SEC measurement, the protein concentration is very low and the term "$2A_2C$" in Eq. (1) could be neglected. Therefore, the M_w and R_g can be calculated according to Eq. (3) by the WinGPC 7.2 software using the Debye method, i.e. regression on the light scattering parameter $R(\theta)/KC$ versus $\sin^2(\theta/2)$

$$\frac{KC}{R(\theta)} = \frac{1}{M_w}\left[1 + \frac{16\pi^2 n_s^2}{3\lambda^2}\langle R_g^2\rangle \sin^2\left(\frac{\theta}{2}\right)\right]. \quad (3)$$

To calculate the molecular weight of peptides, a molecular weight calibration curve was developed, from the average elution volumes of the following standard proteins: BSA (monomer 66.5 kDa, dimer 133 kDa), cytochrome C (12.3 kDa), insulin (5.73 kDa) and vitamin B_{12} (1.36 kDa).

2.4. Dynamic light scattering

The sizes of casein aggregates as a function of hydrolysis time were monitored by dynamic light scattering (DLS). DLS measurements at a 90° scattering angle were carried out by a package containing a MGL-III model 100 mW He–Ne laser, tuned to a wavelength of 532 nm, a computer-controlled BI-200SM goniometer, a BI-9000AT digital correlator and a signal processor (Brookhaven Instruments, Holtsville, NY). Decalin, as an index-matching liquid to reduce flare, was re-circulated through a 47-mm O.D. 0.1-μm pore-size hydrophobic membrane filter (Millipore, Bedford, MA) until visual inspection suggested it was dust-free. Bovine casein was prepared at 2 mg/ml in 20 mM phosphate buffer at pH 8.0 and digested with pancreatin (0.02 mg/ml), at 37 °C, in a borosilicate glass tube (Fisher Scientific, Pittsburgh, PA). The test tubes were placed in the sample holder. The effective hydrodynamic radius (R_h) was calculated from the diffusion coefficient by using the method of cumulants (Brown, Pusey, & Dietz, 1975).

3. Results and discussion

3.1. HPSEC–UV–MALLS–RI analysis of bovine casein

Fig. 1 displays the HPSEC elution profiles of bovine casein from the absorbance at 214 nm (UV), the laser scatter detector at an angle of 90° (MALLS) and the RI signal. In the HPSEC chromatograms of bovine casein, two distinct peaks, at the elution volumes V_e of 5.0 and 6.6 ml, were obtained by using an elutant buffer of water containing 30% ACN. Lucey et al. (2000) reported that the HPSEC of sodium caseinate, using a Superose 6HR 10/30 column, resolved the caseins into a very small peak near the void volume and two large peaks in the elutant buffer of 20 mM imidazole and 50 mM NaCl at pH 7.0. In other previous studies, sodium caseinate had also been resolved into larger and smaller components during HPSEC, by using different elutant buffers (Lorenzen, Schlimme, & Roos, 1998; Lynch, Mulvihill, Law, Leaver, & Horne, 1997).

The molecular weight of peak 1 (elution volume 5.0 ml), determined by MALLS, was approximately 850 kDa (Fig. 1), which could be assigned to casein aggregates. A molecular weight of 30 ± 7 kDa was obtained for peak 2 (Fig. 1), which was close to the molecular weights of individual caseins. The HPSEC–MALLS–RI result indicated that there might be a dynamic system between monomers and aggregate and thus supported the viewpoint of Walstra (1999), that caseins were a type of equilibrium system.

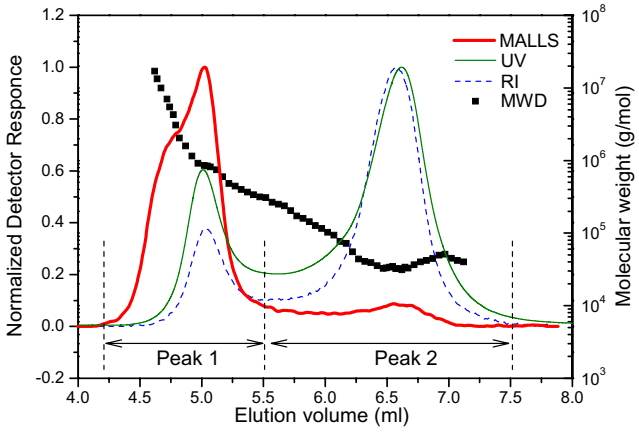

Fig. 1. HPSEC–UV–MALLS–RI elution profile and its calculated molecular weight distribution of bovine casein. The UV detector was monitored at 214 nm and the MALLS response was followed at 90°.

3.2. Changes in the size and molecular weight of casein aggregates during hydrolysis

Limited hydrolysis of bovine casein, by pancreatin, induced the growth in aggregate sizes (Fig. 2). Fig. 2A shows the HPSEC–UV elution profiles of bovine casein pancreatic hydrolysates. As can be seen, bovine caseins were fragmented into different peptides after hydrolysis. The whole hydrolysis process can be divided into two stages. In the first stage, that is from the beginning to about 10 min, the peak areas and elution volumes of casein aggregates both decreased as the reaction proceeded. The longer the hydrolysis time was, the lower were the concentrations of casein aggregates, but the larger were their sizes and molecular weights. The growth in aggregate size can also be differentiated by visual comparison of the UV and MALLS signals. The intensity of the UV signal was proportional to the concentration but that of the MALLS signal was proportional to both concentration and molecular weight (Wyatt, 1993). During this stage, the intensity of the MALLS signal decreased at a slower rate than that of the UV signal (Fig. 2B), also suggesting the increase in the molecular weights of casein aggregates. In the second stage (10–120 min), casein aggregates were almost digested into peptides. There were no significant signals in the aggregate region of the UV and MALLS chromatograms after a 30 min hydrolysis.

Fig. 3 depicts molecular weight distributions of casein aggregates upon hydrolysis by using the analysis of HPSEC–MALLS–RI. During the initial 10 min, the sizes and molecular weights of casein aggregates gradually increased with the hydrolysis time. Table 1 summarised the M_n, M_w, A_2 and $\langle R_g \rangle_z$ values of the aggregates after different hydrolysis times. The aggregates without hydrolysis displayed a M_w of 1.35×10^6 Da, while those formed after 10 min hydrolysis showed a larger M_w of 8.86×10^6 Da. After pancreatic hydrolysis, a significant increase in the $\langle R_g \rangle_z$ of casein aggregates was also observed, from 31.4 to 38.6 nm, which indicated pancreatin-induced aggregation. During the course of hydrolysis, the second virial coefficient (A_2) of casein aggregates were all positive, suggesting that the protein–protein interactions were repulsive (Liu, Cellmer, Keerl, Prausnitz, & Blanch, 2005). After pancreatic hydrolysis of bovine casein, A_2 became less positive indicating the electrostatic repulsion was gradually screened. Such screened repulsion might be correlated with the simultaneous increases in the sizes and molecular weights of the aggregates. Similar behaviour has been previously reported in the case of casein micelles (with calcium phosphate) hydrolysed by plasmin (Crudden et al., 2005). The decrease in the protein repulsion had been considered to be responsible for the formation of aggregates.

Dynamic light scattering (DLS) was used to trace the changes in the aggregate sizes of bovine caseins upon pancreatic hydrolysis. Fig. 4 displays the hydrolysis-time dependence of the effective hydrodynamic radius (R_h) and the light scattering intensity (I_s). The latter is approximately proportional to both the concentration C and the weight-average molecular weight M_w. During the initial 10 min, I_s decreased rapidly while R_h greatly increased,

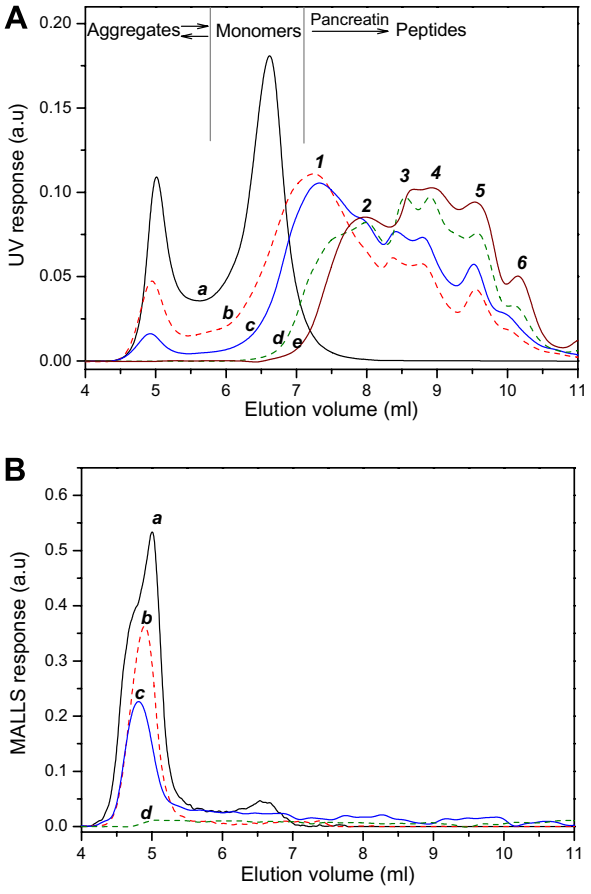

Fig. 2. HPSEC–MALLS profiles of bovine casein undergoing hydrolysis with pancreatin for (a) 0 (control), (b) 5, (c) 10, (d) 30 and (e) 120 min at pH 8 and 37 °C. The UV detector was monitored at 214 nm (A) and the MALLS response was followed at 90° (B).

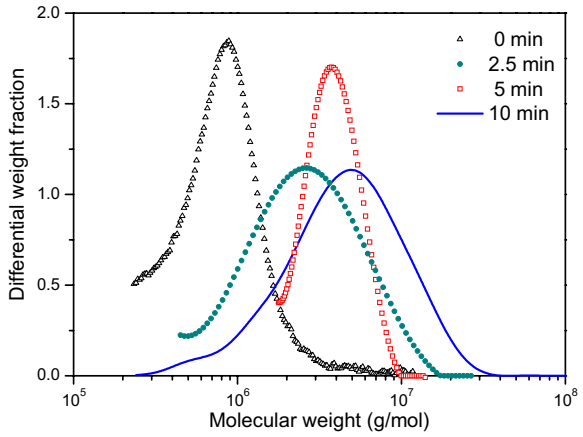

Fig. 3. Molecular weight distributions of casein aggregates, after the hydrolysis with pancreatin for different time, determined by HPSEC–MALLS–RI.

Table 1
Number- (M_n) and weight-average (M_w) molecular weights, second virial coefficient (A_2), and z-average radius of gyration ($\langle R_g \rangle_z$) for casein aggregates (Fig. 3) after different hydrolysis time

Time (min)	M_n (g/mol) × 10^6	M_w (g/mol) × 10^6	M_w/M_n	A_2 (ml mol/g^2) × 10^{-2}	$\langle R_g \rangle_z$ (nm)
0	0.95 ± 0.04	1.35 ± 0.05	1.42	2.04 ± 0.06	31.4 ± 0.6
2.5	1.92 ± 0.14	3.15 ± 0.15	1.64	0.45 ± 0.03	33.7 ± 1.5
5	3.39 ± 0.18	4.94 ± 0.21	1.45	0.37 ± 0.01	36.7 ± 1.9
10	5.16 ± 0.29	8.86 ± 0.32	1.71	0.19 ± 0.01	38.6 ± 2.5

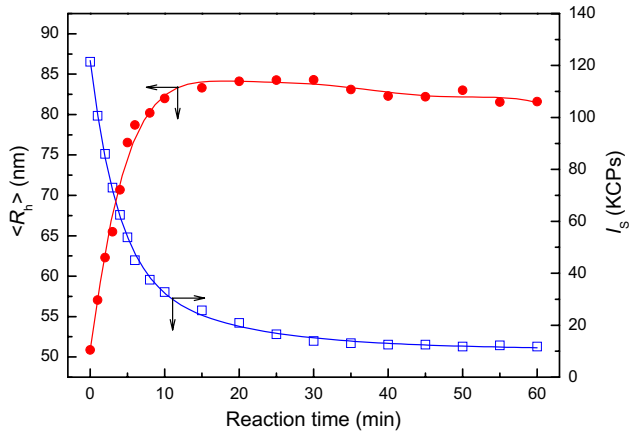

Fig. 4. Effective hydrodynamic radius (R_h) and light scattering intensity (I_s) of bovine casein pancreatic hydrolysates as a function of reaction time. The DLS measurements were performed at 90°. The lines are to guide the eye.

from about 50 to 82 nm, suggesting casein aggregation upon pancreatic hydrolysis. After 10 min, I_s decreased with a slower rate, which indicated a further reduction in the content of aggregates, while the values of R_h became constant at approximately 82 nm.

By using a combination of HPSEC–UV–MALLS–RI and DLS measurements, the increases in aggregate sizes of bovine casein, after limited hydrolysis, were demonstrated. Such changes in aggregate sizes might be elucidated in terms of changes in molecular structure of bovine casein, upon pancreatic digestion. It is now accepted that a casein aggregate (without calcium phosphate) has hydrophobic cores and hydrophilic surfaces and the outer surface of the aggregate is quite diffuse (Chu et al., 1995; Kumosinski, Uknalis, Cooke, & Farrell, 1996). As is well known, pancreatin is a multiple enzyme system, in which trypsin, chymotrypsin and elastase play key roles in the hydrolysis of proteins. Among these enzymes, trypsin and chymotrypsin preferentially hydrolyse peptide bonds with hydrophobic nature (Schellenberger, Turck, Hedstrom, & Rutter, 1993; Schellenberger, Turck, & Rutter, 1994). During the hydrolysis, trypsin and chymotrypsin might cleave the peptide bonds inside the aggregates, which induce the exposure of the hydrophobic cores. By assembling the exposed aggregates, the larger particles could be formed due to hydrophobic attractions and the screened intermolecular repulsion.

3.3. Fractionation and characterisation of bovine casein pancreatic hydrolysates

As displayed in Fig. 2, the light scattering intensity of the resulting peptides was too low to calculate molecular weight with sufficient accuracy. Because the UV detector was more sensitive to small-sized molecules, than the MALLS detector, the resulting peptides could be detected in the UV chromatograms. If the characterisation of product distribution was required, the HPSEC system should first be calibrated. Therefore, we applied the HPSEC columns to separate the standard proteins and casein aggregates according to their hydrodynamic size. The V_e–lg(M) relationship can be observed in Fig. 5. At $V_e \geqslant 5.5$ ml, the calibration curve was $\lg(M) = 8.318–0.5826\,V_e$ (V_e in ml, M in g/mol) via standard proteins. At $V_e < 5.5$ ml, the SEC columns were calibrated with the V_e and M of casein aggregates, as determined by HPSEC–MALLS–RI, at their peak positions. Because the large molecules would elute near the void volume of the columns, the value of lg(M) was exponentially increased with decreasing elution volume while $V_e < 5.5$ ml.

We next examined the molecular weight distribution of bovine casein pancreatic hydrolysates by using the V_e–lg(M) relationship. As shown in Fig. 2A, the peptide chains of individual caseins were cleaved into various concentrative fragments including macropeptides (20.0–7.0 kDa, peak 1; 7.0–3.0 kDa, peak 2), polypeptides (3.0–2.0 kDa, peak 3; 2.0–0.8 kDa, peak 4; 0.8–0.4 kDa, peak 5) and oli-

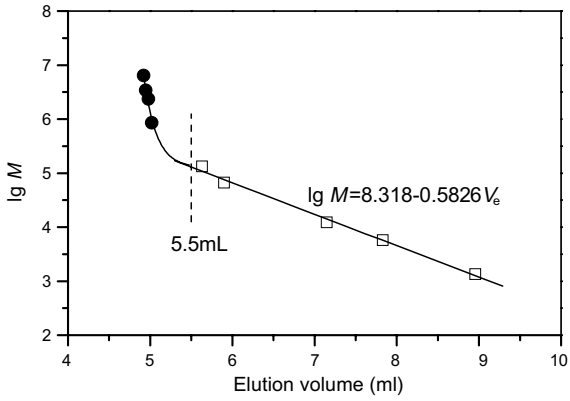

Fig. 5. The calibration curves of molecular weight (M) and the elution volume (V_e) by using (□) the standard proteins and (•) the casein aggregates after different hydrolysis times.

gopeptides (<0.4 kDa, peak 6). These fractions all appeared in the initial stage of hydrolysis (5 min) but the changes in their concentrations displayed different traces as the reaction proceeded.

The HPSEC–UV elution profiles in Fig. 2A were recorded at 214 nm, which were generally applied for the detection of proteins and peptides. The absorption at this wavelength was mainly caused by peptide bonds and therefore the concentrations of small peptides could be underestimated. However, it was difficult to use a correction factor, since the exact composition of each molecular weight fraction was not known. Therefore, the peak area of UV chromatograms at 214 nm was applied to examine the changes in the protein/peptide concentration in spite of its imperfection.

Fig. 6 shows the release kinetic of five molecular weight fractions during pancreatic hydrolysis of bovine casein. It can be seen from Fig. 6 that: (1) the reductions in both casein aggregates and monomers were rapid in the initial stage of hydrolysis (DH < 6%) but slower subsequently. The original substrates were almost digested at the DH value of 12%, (2) the macropeptides (20.0–3.0 kDa, peaks 1 and 2 in Fig. 2A), which might contain various antimicrobial peptides, showed a fast formation at DH < 6% and then a slower decrease. Above 6%, the intermediate fraction would still be generated and converted to the smaller peptides, (3) the polypeptides (3.0–0.4 kDa, peaks 3, 4 and 5 in Fig. 2A) might be a power source of antihypertensive peptides. There was a rapid increase in these peak areas at DH < 6% which became subsequently slower, (4) the oligopeptides (<0.4 kDa, peak 6 in Fig. 2A) should be mainly composed of di- and tri-peptides, which possessed a high intestinal absorption rate. The concentration of this fraction, which might be underestimated due to its lower absorbance at 214 nm, displayed a continuous increase during the course of hydrolysis. The similar trend for the HPSEC profiles of the hydrolysates was previously observed in the case of bovine casein digested with trypsin (Qi, He, & Shi, 2003).

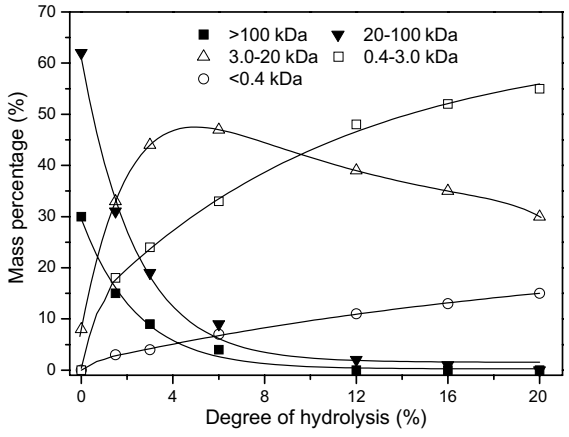

Fig. 6. Changes in the mass percentages of different protein/peptide fractions, based on molecular weight intervals of the HPSEC profiles, during pancreatic hydrolysis of bovine casein. The lines are to guide the eye.

It should be noted that the concentration ratio of casein aggregates to individual caseins was almost constant, at approximately 1:(2–2.5), during the initial 10 min hydrolysis, as depicted by HPSEC–UV chromatograms in Fig. 2A. Such distribution of aggregates and monomers suggested that the caseins in the solutions exist as a dynamic system of casein aggregates and monomers.

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