JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5529–5534 5529
DOI:10.1021/jf803313h

Pressure-Aided Proteolysis of β-Casein

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β-Casein, which is present in the form of micelles at atmospheric pressure, has been hydrolyzed during pressure treatment to improve the accessibility of the protein. Two proteolytic enzymes with different specificities were used. Trypsin was aimed at mainly hydrolyzing hydrophilic segments of β-casein and chymotrypsin at hydrolyzing hydrophobic segments of β-casein. Measurements on aggregation during hydrolysis at atmospheric pressure showed that probably not micelle disruption, but disruption of much larger aggregates, occurs in the process. Peptide profiles were measured via reversed-phase chromatography. Measurements on enzyme activity after pressure treatments showed that trypsin was inactivated by pressure, which could explain all differences in peptide profiles compared to atmospheric experiments. Pressure did not influence the reaction mechanism, probably because the hydrophilic part of β-casein is sufficiently accessible. However, chymotryptic proteolysis under pressure yielded new peptides that could not be explained by a change in enzyme activity. Here, pressure altered the mechanism of hydrolysis, by changing either enzyme specificity or substrate accessibility, which led to different peptides that can have different properties.

KEYWORDS: Trypsin; chymotrypsin; micelles; high hydrostatic pressure; hydrophobicity; peptides; β-casein

INTRODUCTION

Enzymatic hydrolysis is frequently used to improve the functional and nutritional properties of food proteins. The use of pressure may alter the process of hydrolysis and lead to different peptides that can have reduced bitterness and better emulsifying properties or cause less allergenic reactions (*1*). In the food industry, where many existing products and production methods are already patented, a method such as pressurization that may produce different new peptides is highly interesting. The most studied protein for pressure-aided hydrolysis is probably β-lactoglobulin (*2*−*10*). It is one of the proteins responsible for intolerance and/or allergenic response to cow's milk in humans. Other proteolytic reactions under pressure that have been studied are the hydrolysis of ovalbumin (*11*) and soybean proteins (*12*). However, because many studies are not also conducted in time, it is difficult to conclude if product differences are caused by the involvement of a different hydrolysis mechanism or by a change in the reaction rate.

Pressure can be used to increase the surface hydrophobicity of proteins (*13*−*17*) and can therefore aid in proteolysis when the hydrophobic parts of the protein need to be accessed. An interesting protein in this respect is β-casein, which is one of the principal components (about 35% w/w) of the casein fraction of bovine milk proteins and one of the most abundant proteins in food products (*18*, *19*). The unique feature of β-casein is that it lacks tertiary structure in its native state. The protein has a short N-terminal part that mostly consists of hydrophilic residues and harbors nearly all of the protein electrostatic charges and a long C-terminal part that is mainly hydrophobic and is characterized by almost complete lack of charges. Due to this amphiphilic nature the protein undergoes self-association and forms micellar-like aggregates in aqueous media. The association of β-casein is reversible, dependent on temperature, pressure, pH, ionic strength, and protein concentration (*20*, *21*).

High-pressure studies show that associated β-casein molecules partially dissociate with increasing pressure up to 150 MPa and then reassociate under higher pressures up to 300 MPa (*21*). Similar results are found for trypsin-treated β-casein solutions. The minimum in associated state, in this case measured by the turbidity, can differ and is dependent on the extent of proteolysis. In a 24% (proteolysis/maximal proteolysis) degraded sample the minimum is lowered to around 90 MPa. Next, the minimum increases again. At around 50% hydrolysis the minimum turbidity value is back at 150 MPa and further increases with the degree of hydrolysis (*22*).

We have used proteolysis to probe the effects of high pressure on β-casein micellar state. We have chosen two gastrointestinal proteinases, trypsin and α-chymotrypsin, that have distinct specificities but can be used under similar conditions (pH 7.8 and 37 °C). Chymotrypsin hydrolyzes hydrophobic protein segments with aromatic and other bulky hydrophobic side chains, including tryptophan, tyrosine, phenylalanine, leucine, and methionine, which are cleaved at their C-terminal sides. Due to

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 Published on Web 05/22/2009

the hydrophobicity of its major target residues that mostly lie in the inner part of the protein, micelle formation or disruption is expected to have an influence on hydrolysis. In contrast, trypsin is especially hydrolyzing hydrophilic parts, which lie at the outer part of the protein, facing the solvent. It predominantly cleaves proteins at the C-terminal side of lysine and arginine, except when either is followed by a proline.

The two enzymes of our choice are sufficiently barostable. According to high-pressure studies based on fluorescence and Fourier transformed infrared spectroscopy (FTIR) at 0 °C and pH 7.3, trypsin is pressure stable up to 500 MPa (*23*). A study by Mozhaev et al. showed that chymotrypsin was activated by pressure. Above 360 MPa, the enzymatic activity decreased again due to pressure-induced denaturation (*24*).

The aim of this paper is to provide a better understanding of the effect of high pressures on β-casein micellar state and its susceptibility to proteolysis. For this purpose, we examined peptide profiles influenced by hydrolysis time and pressure. We investigated the effects of two different enzymes that mainly act on the hydrophobic or hydrophilic segments of the protein. For further insight into the influence of hydrophobicity, we also study protein aggregation in time.

MATERIALS AND METHODS

Materials. β-Casein from bovine milk (C6905), tosyl phenylalanyl chloromethyl ketone (TPCK) treated bovine pancreas trypsin (T1426), and *N*-succinyl L-phenylalanine *p*-nitroanilide (Suc-Phe-pNA) were purchased from Sigma Chemical Co. (St Louis, MO). Bovine pancreas α-chymotrypsin (1.02307) and 5′-benzoyl DL-arginine *p*-nitroanilide hydrochloride (DL-BAPA) were purchased from Merck (Darmstadt, Germany).

Degree of Hydrolysis. First experiments at atmospheric pressure were conducted in a pH-stat to determine the appropriate amount of enzyme to be added to the β-casein solution. Ten grams per liter β-casein in 126 mM NaCl was stirred overnight at 5 °C and preheated at 37 °C for 20 min. The pH was adjusted to pH 7.8 for another 20 min before addition of the enzyme solution. During hydrolysis, the reaction mixture was maintained at pH 7.8 by the addition of a 0.4 M NaOH solution in a pH-stat (719 S Titrino, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). NaOH addition was recorded in time and used to calculate the degree of hydrolysis (*25*).

Particle Size of the β-Casein Micelles. The average particle size of the β-casein micelles was determined by photon correlation spectroscopy (PCS) on the Zetasizer Nano ZS at 37 °C (Malvern Instruments, Malvern, U.K.). This particle sizer uses back scattering with an angle of 173°, which also enables measurement in undiluted solutions, preventing dissociation of the β-casein micelles due to dilution effects.

Three light scattering measurements of 60 s were carried out for each sample [refractive index of the outer phase (water), 1.330; viscosity of the outer phase, 0.6864; and laser wavelengt, 632.8 nm]. The results, expressed in nanometers, are determined from the intensity distribution curves.

Enzymatic Hydrolysis of β-Casein at Atmospheric Pressure. Ten grams per liter β-casein in 50 mM Tris and 100 mM NaCl in Milli-Q was stirred overnight at 5 °C. The Tris buffer was made to pH 7.8 at 37 °C. Enzyme was added to the reaction mixture and the reaction continued for 5 days. Samples were taken in time. The amount of enzyme added was based on previous pH-stat measurements such that in 3 h, half of the maximal degree of hydrolysis was reached. The final concentrations of chymotrypsin or trypsin in the reaction mixture were 0.18 and 0.001 (w/w), respectively. The reaction mixture was kept at 37 °C in a water bath, and samples were taken in time for peptide analysis on reversed-phase chromatography. The complete experiment was repeated to validate results.

Enzymatic Hydrolysis of β-Casein at High Pressure. Reactions under high pressure were performed in a multivessel high-pressure apparatus (Resato FPU 100-50, Resato International B.V., Roden, The Netherlands). The reaction mixtures were pipetted in polyethylene bags, which were closed by sealing. As pressure medium a glycol mixture was used. The vessels were individually pressurized by an electronic driven hydraulic pump and a high-pressure intensifier unit. The pressurization rate was approximately 200 MPa min^{-1}. The vessels were kept at a constant temperature by a water bath set at 37 °C. During pressurization, the temperature in the vessels increased due to adiabatic heating, but did not exceed 44 °C. Temperature was back to 37 °C within 10 min. The reaction was kept under pressure for 3 h, which was the time required to reach half the maximal degree of hydrolysis at atmospheric pressure. Samples were taken for reversed-phase chromatography and enzyme activity tests. The complete experiment was repeated to validate results.

Enzyme Activity. Enzyme assays were conducted with synthetic substrates at room temperature, in 50 mM Tris-HCl buffer containing 100 mM $CaCl_2$, pH 8.1. Trypsin activity was assayed using DL-BAPA (*26*). Four milligrams of DL-BAPA was dissolved in 100 μL of DMSO and diluted with 10 mL of the buffer. From the reaction mixture, a sample volume of 50 μL was incubated with 200 μL of substrate solution. Chymotrypsin activity was assayed using Suc-Phe-pNA (*27*, *28*). Ten milligrams of Suc-Phe-pNA was dissolved in 1 mL of acetonitrile, to which 9 mL of the buffer was added. From the reaction mixture, a sample volume of 20 μL was incubated with 180 μL of substrate solution. In both assays the absorbance of *p*-nitroanilide produced in time was measured in a microtiter plate reader at 405 nm.

Analytical Reversed-Phase Chromatography. Samples from the reaction mixture were inactivated by the addition of 1.0 M HCl to reach a final sample pH of 2. Peptides were separated on an analytical Vydac C18 column (218MS52; 250 × 2.1 mm; bead diameter, 5 μm; porosity, 300 nm; Dionex, Sunnyvale, CA) by HPLC (Thermo Separation Products Inc., San Jose, CA) with Chromeleon software. The flow rate was 0.2 mL min^{-1} and the column temperature, 20 °C. Eluent A was 0.07% (v/v) TFA in 5% (v/v) acetonitrile, and eluent B was 0.05% (v/v) TFA in 80% acetonitrile. Samples to be analyzed were further diluted with eluent A to reach a final proteinaceous concentration of 1 mg mL^{-1}. A volume of 20 μL was injected onto the column. After 10 min of isocratic elution with eluent A, further elution was obtained with a linear gradient from 0 to 60% eluent B in 90 min and then from 60 to 100% eluent B in 1 min and 100% B for 10 min. Detection was performed at 214 nm.

Electrospray Mass Spectrometry. Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ Deca XP MAX, Thermo Finnigan, San Jose, CA) connected to the reversed phase chromatography unit. It was operated in the positive mode using a spray voltage of 2 kV and a capillary temperature of 200 °C. The capillary voltage was set at 45 kV and the tube lens voltage at 35 kV. Mass spectra were collected in a full mass scan, followed by a zoom scan and a MS/MS scan of the most intense ion in a window of m/z 1.5−2 and a 30−35% relative collision energy. The apparatus and data were controlled by Xcalibur software. The accuracy of the mass determination is ± −0.3 Da. The theoretical masses of peptides were calculated using the program Protein Prospector MS Digest v 4.27.2 by P. R. Baker and K. R. Clauser (http://prospector.ucsf.edu).

RESULTS

Micelle Size during Proteolysis at Atmospheric Pressure. Changes in the hydrodynamic diameter of the β-casein micelle in solution at different times are depicted in **Figure 1** for tryptic and in **Figure 2** for chymotryptic hydrolyzed β-casein. As a blank β-casein without enzyme was incubated at 37 °C. The blank showed a gradual, but very slow, increase of size in time (results not shown). Starting size of the β-casein micelle was 31 nm, increasing to 34 and 36 nm after 2.5 and 5 h, respectively.

When trypsin was added to the β-casein, the micelle size increased to approximately 150 nm after 2.5 h and remained stable at that value for several hours. After 5 h, the size increased to reach a maximum around 2000−3000 nm after half a day. Next, micelle size decreased again to below 200 nm after 1 day. In that period a second peak developed (shown as the diamonds in **Figure 1b**) and disappeared again into the main peak.

The first measurement after the chymotrypsin addition (sample 0) showed immediate and almost maximal aggregation.

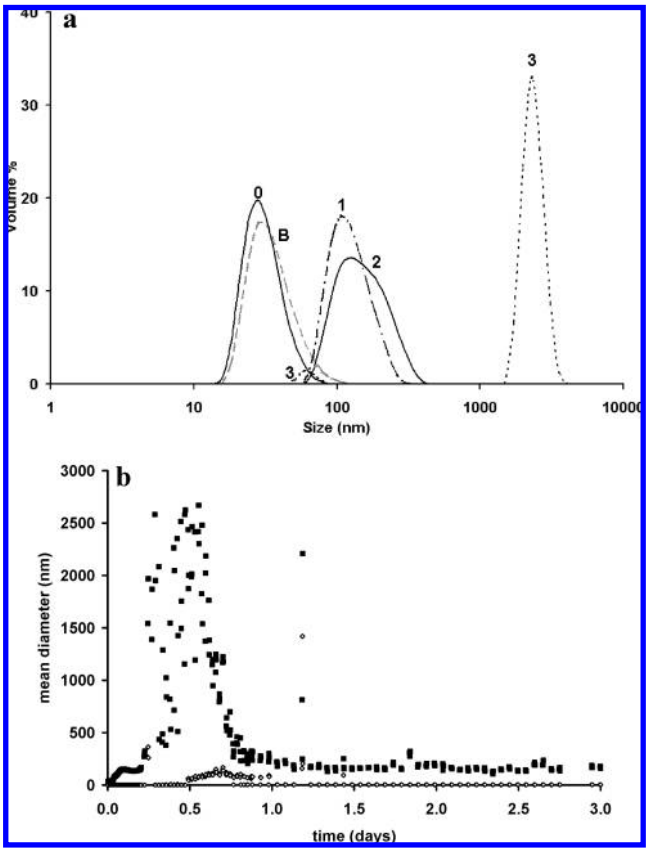

Figure 1. (**a**) Size distribution of micelles during proteolysis of β-casein with trypsin at 0.1 MPa and 37 °C. B, blank, no enzyme added; 0, t = 1 min; 1, t = 2.5 h; 2, t = 5 h; 3, t = 12 h of hydrolysis. (**b**) Mean diameter in time of the (■) major peak and (◇) smaller second peak.

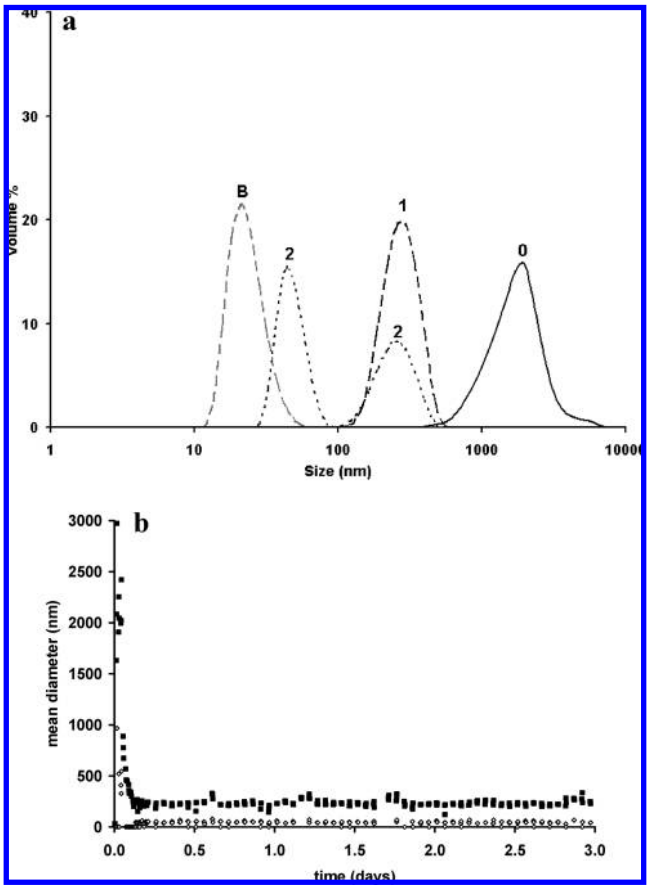

Figure 2. (**a**) Size distribution of micelles during proteolysis of β-casein with chymotrypsin at 0.1 MPa and 37 °C. B, blank, no enzyme added; 0, t = 1 min; 1, t = 2.5 h; 2, t = 5 h of hydrolysis. (**b**) Mean diameter in time of the (■) major peak and (◇) smaller second peak.

The aggregates had a diameter between 2000 and 3000 nm. This aggregation may be caused by the relatively large amount of chymotrypsin (2% of total protein) that has been added to the mixture. The addition is larger than that of trypsin (0.01% of total protein). The first measurement is not a blank because it takes at least 1 min to complete a measurement and the aggregation is very fast. Next, the aggregates' size slowly decreased to stabilize after approximately 2.5 h at around 240 nm. Between 2.5 and 5 h a second peak appeared that had a smaller size (around 50 nm). After 5 h, the size of the aggregates did not change any further.

Proteolysis in Time: Trypsin. Proteolysis by trypsin was measured in time. In **Figure 3** the resulting peptide profiles are depicted at different times: immediately after addition of the enzyme (t = 0), after 30 min, and after 3 h at half the maximal degree of hydrolysis. The peptide profiles continually evolve in time. The peptide profile of the trypsin-hydrolyzed β-casein does not contain many peptides, which is consistent with the low maximal degree of hydrolysis (DH_{max}) of 7.2% and the expected maximum of 16 peptides. Many peaks are also to the right-hand side of the chromatogram, indicating large and/or hydrophobic peptides. We were able to identify many of the peptide peaks after 3 h of hydrolysis at atmospheric pressure, which were consistent with literature data (*29*). The results are given in **Table 1**. We found an additional peak (f184−209, peak j), but the Girardet spectrum does not show results at high retention times. This peak is, however, yet obtained by Briand et al. (*30*). The important difference between the Girardet spectrum and ours is the occurrence of one peak (f33−97, peak h) instead of two (f33−48 and f49−97) or even three peaks (f33−48, f49−68, and f69−97), which indicates that this peptide is cleaved at a very late stage

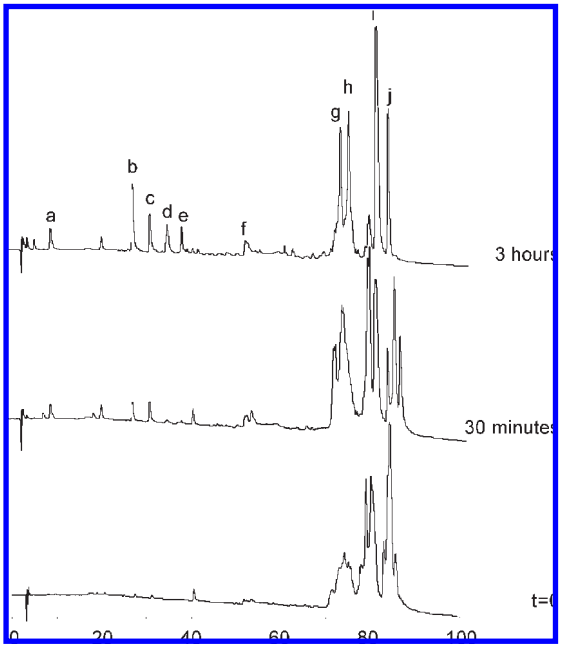

Figure 3. Peptide profiles in time of β-casein hydrolysis with trypsin at 0.1 MPa and 37 °C. The letters correspond to those in **Table 1**.

in hydrolysis. The slow hydrolysis of the Asn68−Ser69 bond was also found by Girardet when β-casein was hydrolyzed by trypsin on an oil−water interface. On the basis of a hypothetical model of

Table 1. MS Data of β-Casein Tryptic Digest at 0.1 MPa and 37 °C

peak	measured mass	possible fragment
a	645.3	100−105
b	829.4	177−183
c	779.2	170−176
d	1012.4	106−113
e	747.1	108−113
f	3122.0	1−25
g	7401.1	related to peak h
h	7361.6	33−97
i	6361.9	114−169
j	2909.4	

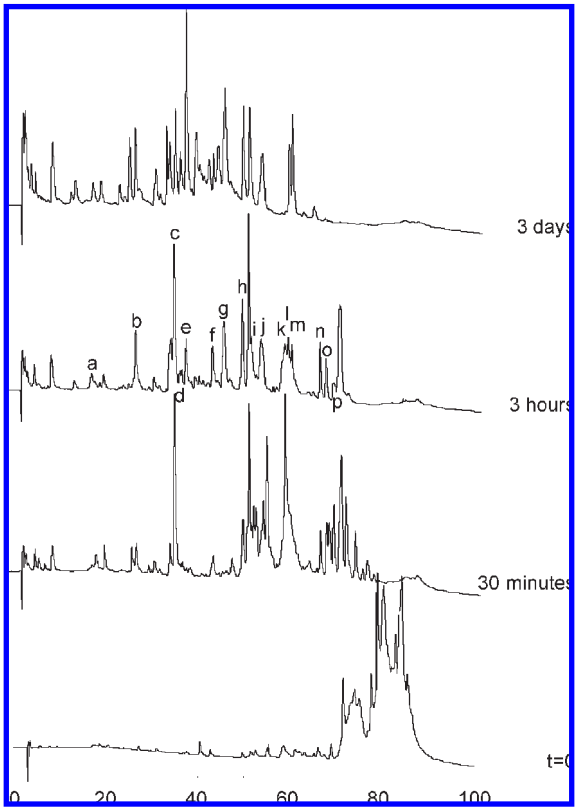

Figure 4. Peptide profiles in time of β-casein hydrolysis with chymotrypsin at 0.1 MPa and 37 °C. The letters correspond to those in **Table 2**.

the tertiary structure of β-casein, they located the bond in a distinct hydrophobic region, suggesting that this hydrophobic region is less accessible under pressure.

Proteolysis in Time: Chymotrypsin. Proteolysis by chymotrypsin was measured in time. In **Figure 4** the resulting peptide profiles are depicted at several different times: immediately after addition of the enzyme (t = 0), after 30 min, and after 3 h at half the maximal degree of hydrolysis, and after 3 days. When compared to the t = 0 sample of the tryptic digest, several additional peaks are already visible, indicating a relatively fast reaction. The peptide profiles continually evolve in time. The peptide profile of β-casein hydrolyzed by chymotrypsin consists of many peptides. This is consistent with the large degree of hydrolysis (DH_{max} = 42%) that can be achieved by using chymotrypsin and the expected maximum of 88 peptides. The 3 day peptide profile is similar to the 2 day peptide profile (not shown), indicating that full hydrolysis, or at least equilibrium, was reached within 2 days. We were unable to identify all of the peptides via MS due to a lack of separation. Peptides that were identified are listed in **Table 2**. In the literature, we could not find any results on identified peptide

Table 2. MS Data of β-Casein Chymotryptic Digest at 0.1 and 37 °C and Its Evolution at 600 MPa and 37 °C

peak	measured mass	possible fragment	at 600 MPa
a	663.3	120−125	peak almost disappeared
b	829.3	177−183	peak almost disappeared
c	1012.9 and 532.3	106−113 and 140−143	similar
d	993.8 and 407.1	194−202 and 191−193	one peak disappeared
e	747.4	108−113	peak disappeared
f	903.3	126−133	similar
g	750.3	114−119	peak lower
h	1395.6 and 820.4	114−125 and 184−190	peak lower
i	688.5	134−139	similar
j	1745.8	106−119 or 177−191	higher peak
k	2150.1	145−163	similar
l	2282.1	144−163 ?	similar
m	741.4	203−209	peak almost disappeared
n	1717.9	194−209	peak lower
o	1881.0	193−209	peak lower
p	1994.1	192−209	Peak lower

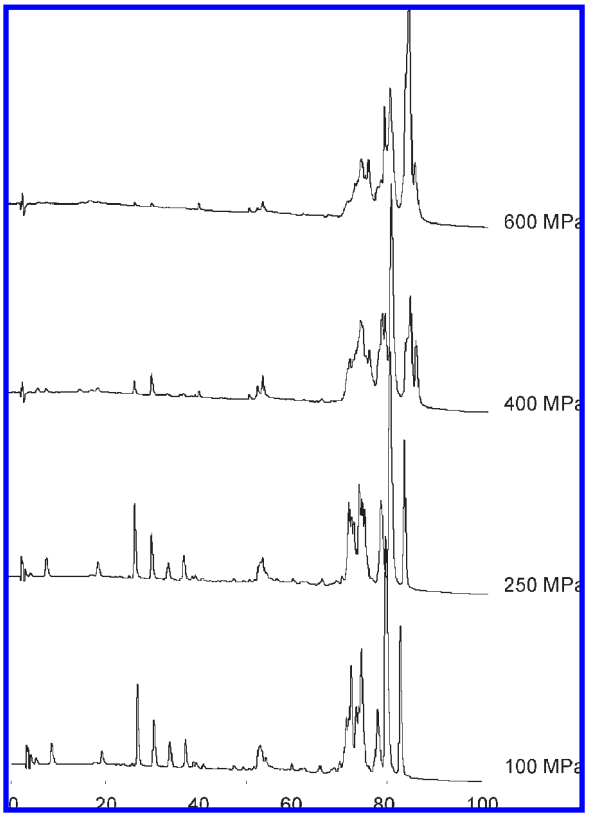

Figure 5. Peptide profiles at different pressures at 37 °C after 3 h of β-casein hydrolysis with trypsin.

profiles with chymotryptic hydrolysis of β-casein, which agreed with our difficulties in getting a well-resolved peptide profile.

Proteolysis under Pressure: Trypsin. Proteolysis of β-casein by trypsin was also measured under pressure. In **Figure 5** the resulting peptide profiles are depicted after 3 h of hydrolysis. The profiles show fewer and larger peptides with increasing pressure. The profile at 600 MPa looks quite similar to the blank profile, indicating that the enzyme is inactivated. The other profiles look similar to the profiles from the time experiment, only with less hydrolysis. The results can, therefore, be explained by a decrease in enzyme activity under pressure. Trypsin specificity and selectivity that determine product formation and (intermediate) substrate choice, as well as substrate accessibility for trypsin, were not modified under high pressure.

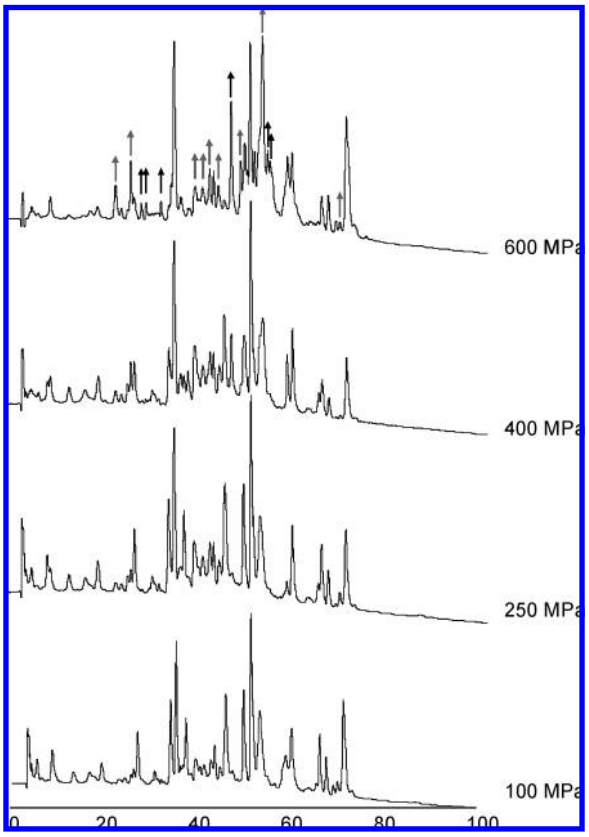

Figure 6. Peptide profiles at different pressures at 37 °C after 3 h of β-casein hydrolysis with chymotrypsin. Arrows indicate the peaks that increased in size compared to the same peak at atmospheric pressure. Black arrows are peaks that also do not appear at a later stage of hydrolysis at atmospheric conditions; gray peaks do appear at a later stage of hydrolysis at atmospheric conditions.

Proteolysis under Pressure: Chymotrypsin. Proteolysis of β-casein by chymotrypsin was also measured under pressure. In **Figure 6** the resulting peptide profiles are depicted after 3 h of hydrolysis. The profiles do not show obvious differences like the tryptic digest. Peptides are still being formed, even at 600 MPa, suggesting that inactivation of the enzyme is not severe. However, many of the peaks that occur at atmospheric pressure decrease under pressure and more importantly some other, new peaks are formed. This indicates a different hydrolytic mechanism that may be due to either a difference in accessibility of the β-casein or a difference of chymotrypsin specificity or selectivity. The evolution at 600 MPa of the identified peaks at atmospheric pressure is included in **Table 2**. Only one peptide (identified as either f106−119 or f177−191, peak j) shows a distinct increase at 600 MPa. All other peptides have a lower or similar response. New peaks and peaks that showed remarkable increase are marked with arrows in **Figure 6**. Unfortunately, we were unable to identify any of them. The new peaks were also compared to peptide profiles that were measured after prolonged hydrolysis of the β-casein, at atmospheric pressure. Peptides that were also not visible on the peptide profile at 6, 12, 24, and 48 h are shown with black arrows in **Figure 6**. These are clearly new peaks. Other peaks that were increased by pressure, but also occurred after prolonged reaction at atmospheric conditions, are marked in gray. These peaks may have occurred after 3 h at 600 MPa because of an increase in enzyme activity under pressure.

Residual Enzyme Activity after Pressure Treatment. In **Table 3** the residual enzyme activities after 3 h of reaction at different pressures are given. Storage on ice at atmospheric pressure is taken as a blank. Already at atmospheric pressure and 37 °C, the enzymes start to denature irreversibly, as shown by the reduced activity after 3 h at 37 °C compared with that after 3 h at 0 °C. Chymotrypsin is, however, stabilized by pressure as can be seen from the residual activity at 250 MPa, which is even higher than the blank. Higher pressure inactivates both enzymes, and at 600 MPa residual activity is reduced considerably. For trypsin these results coincide with the resulting peptide profiles at 600 MPa, which also show larger peptides and thus less hydrolysis than the profiles at lower pressures. The chymotryptic peptide profile at 600 MPa shows far more hydrolysis than can be expected from the inactivation experiments. A similar degree of hydrolysis can, however, be obtained when enzyme inactivation is compensated by an increase in enzyme activity under pressure. This may not be unlikely because literature studies show that the maximal rate of hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide by chymotrypsin at 37 °C and 470 MPa is roughly 10 times the maximal rate of hydrolysis under the same, but atmospheric, conditions (*24*).

Table 3. Residual Enzyme Activity after 3 h

conditions	trypsin (%)	chymotrypsin (%)
0 °C, 0.1 MPa	100	100
37 °C, 0.1 MPa	83	88
37 °C, 250 MPa	74	106
37 °C, 400 MPa	59	75
37 °C, 600 MPa	29	23

DISCUSSION

Hydrolyses of β-casein at atmospheric pressure showed major aggregation during hydrolysis with both enzymes. Chymotryptic hydrolysis of β-casein instantaneously yielded large aggregates, whereas tryptic aggregation was much slower, with a maximum at 12 h. Because the hydrophobic part is responsible for the association of β-casein (*31*), the faster aggregation of the chymotrypsin-treated β-casein suggests an earlier exposure of the hydrophobic part of the protein. The trypsin may first cleave off part of the hydrophilic segment before remaining hydrophobic clusters become exposed and aggregation starts. Research by Caessens et al. showed that during β-casein hydrolysis by plasmin, a proteolytic enzyme with trypsin-like specificity, a precipitate was formed that consisted of the hydrophobic part of the protein (*32*).

These observations on aggregation at atmospheric pressure show that in our pressure experiments, it most probably was not standard micelles that were disrupted for easier hydrolytic attack, but much larger aggregates. Turbidity studies on trypsin-treated β-casein solutions by Ohmiya et al. show that disruption of such a system is possible by pressure (*22*).

Pressure can be used to increase the surface hydrophobicity of proteins by exposing embedded hydrophobic parts of the protein (*13*−*17*). It can therefore aid proteolysis when the hydrophobic parts of the protein need to be accessed. Chymotryptic hydrolysis of β-casein was affected by the pressure treatment. Peptides that did not occur under atmospheric conditions were formed at 600 MPa. Formation of these new peptides and reduction of other peptides can be caused by a change in enzyme specificity and/or a change in substrate accessibility. On the other hand, pressure did not seem to affect the specificity or the selectivity of trypsin during hydrolysis of β-casein as the same peptides were produced over the different pressures applied. Only the activity of trypsin was reduced upon pressurization. The tryptic results agree with a study on β-casein hydrolysis by plasmin, which showed similar peptide profiles at 0.1 and 800 MPa (*33*).

In general, pressure-aided proteolysis may be a good tool when hydrophobic parts of the protein need to be accessed. From a practical point of view it may be a method to change peptide profiles or more fundamentally a tool to study protein and peptide interactions. In this study we used proteolysis to probe the effects of high pressure on β-casein solutions and were able to produce peptides that do not occur at atmospheric conditions.

ACKNOWLEDGMENT

We thank Ariette Matser from Agrotechnology and Food Innovations (WUR) for making the high-pressure equipment available for our studies.

LITERATURE CITED

(1) Kilara, A.; Panyam, D. Peptides from milk proteins and their properties. *Crit. Rev. Food Sci. Nutr.* **2003**, *43* (6), 607–633.
(2) Dufour, E.; Hervé, G.; Haertle, T. Hydrolysis of β-lactoglobulin by thermolysin and pepsin under high hydrostatic-pressure. *Biopolymers* **1995**, *35* (5), 475–483.
(3) Bonomi, F.; Fiocchi, A.; Frokiaer, H.; Gaiaschi, A.; Iametti, S.; Poiesi, C.; Rasmussen, P.; Restani, P.; Rovere, P. Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. *J. Dairy Res.* **2003**, *70* (1), 51–59.
(4) Bonomi, F.; Iametti, S.; Rasmussen, P.; Restani, P.; Rovere, P. Advances in the combined application of enzymatic and physical treatments for reducing food allergenicity. *High Pressure Res.* **2000**, *19* (1−6), 565–571.
(5) Chicon, R.; Lopez-Fandino, R.; Quiros, A.; Belloque, J. Changes in chymotrypsin hydrolysis of β-lactoglobulin A induced by high hydrostatic pressure. *J. Agric. Food Chem.* **2006**, *54*, 2333–2341.
(6) Hayashi, R.; Kawamura, Y.; Kunugi, S. Introduction of high pressure to food processing: preferential proteolysis of β-lactoglobulin in milk whey. *J. Food Sci.* **1987**, *52* (4), 1107–1108.
(7) Stapelfeldt, H.; Petersen, P. H.; Kristiansen, K. R.; Qvist, K. B.; Skibsted, L. H. Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.* **1996**, *63* (1), 111–118.
(8) Vanwillige, R. W. G.; Fitzgerald, R. J. Tryptic and chymotryptic hydrolysis of β-lactoglobulin-A, β-lactoglobulin-B and β-lactoglobulin-AB at ambient and high-pressure. *Milchwissenschaft−Milk Sci. Int.* **1995**, *50* (4), 183–186.
(9) Penas, E.; Snel, H.; Floris, R.; Prestamo, G.; Gomez, R. High pressure can reduce the antigenicity of bovine whey protein hydrolysates. *Int. Dairy J.* **2006**, *16* (9), 969–975.
(10) Penas, E.; Prestamo, G.; Baeza, M. L.; Martinez-Molero, M. I.; Gomez, R. Effects of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *Int. Dairy J.* **2006**, *16* (8), 831–839.
(11) Quiros, A.; Chichon, R.; Recio, I.; Lopez-Fandino, R. The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chem.* **2007**, *104* (4), 1734–1739.
(12) Penas, E.; Prestamo, G.; Gomez, R. High pressure and the enzymatic hydrolysis of soybean whey proteins. *Food Chem.* **2004**, *85* (4), 641–648.
(13) Liu, X. M.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. *Innovative Food Sci. Emerging Technol.* **2005**, *6* (3), 310–317.
(14) Altuner, E. M.; Alpas, H.; Erdem, Y. K.; Bozoglu, F. Effect of high hydrostatic pressure on physicochemical and biochemical properties of milk. *Eur. Food Res. Technol.* **2006**, *222* (3−4), 392–396.
(15) Knudsen, J. C.; Lund, M.; Bauer, R.; Qvist, K. B. Interfacial and molecular properties of high-pressure-treated β-lactoglobulin B. *Langmuir* **2004**, *20* (6), 2409–2415.
(16) Lullien-Pellerin, V.; Popineau, Y.; Meersman, F.; Morel, M. H.; Heremans, K.; Lange, R.; Balny, C. Reversible changes of the wheat gamma 46 gliadin conformation submitted to high pressures and temperatures. *Eur. J. Biochem.* **2001**, *268* (22), 5705–5712.
(17) Gaucheron, F.; Famelart, M. H.; Mariette, F.; Raulot, K.; Michel, F.; LeGraet, Y. Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. *Food Chem.* **1997**, *59* (3), 439–447.
(18) Wong, D. W. S.; Camirand, W. M.; Pavlath, A. E. Structures and functionalities of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (8), 807–844.
(19) Southward, C. R. Manufacture and applications of edible casein products. 1. Manufacture and properties. *N. Z. J. Dairy Sci. Technol.* **1985**, *20* (2), 79–101.
(20) O'Connell, J. E.; Grinberg, V. Y.; de Kruif, C. G. Association behavior of β-casein. *J. Colloid Interface Sci.* **2003**, *258*, 33–39.
(21) Payens, T. A. J.; Heremans, K. Effect of pressure on temperature-dependent association of β-casein. *Biopolymers* **1969**, *8* (3), 335–345.
(22) Ohmiya, K.; Kajino, T.; Shimizu, S.; Gekko, K. Effect of pressure on the association states of enzyme-treated caseins. *Agric. Biol. Chem.* **1989**, *53* (1), 1–7.
(23) Ruan, K. C.; Lange, R.; Meersman, F.; Heremans, K.; Balny, C. Fluorescence and FTIR study of the pressure-induced denaturation of bovine pancreas trypsin. *Eur. J. Biochem.* **1999**, *265* (1), 79–85.
(24) Mozhaev, V. V.; Lange, R.; Kudryashova, E. V.; Balny, C. Application of high hydrostatic pressure for increasing activity and stability of enzymes. *Biotechnol. Bioeng.* **1996**, *52* (2), 320–331.
(25) Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science: London, U.K., 1986.
(26) Erlanger, B. F.; Cohen, W.; Kokowsky, N. Preparation and properties of 2 new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **1961**, *95* (2), 271–278.
(27) Delmar, E. G.; Largman, C.; Brodrick, J. W.; Geokas, M. C. Sensitive new substrate for chymotrypsin. *Anal. Biochem.* **1979**, *99* (2), 316–320.
(28) Erlanger, B. F.; Edel, F.; Cooper, A. G. Action of chymotrypsin on 2 new chromogenic substrates. *Arch. Biochem. Biophys.* **1966**, *115* (1), 206–210.
(29) Girardet, J. M.; Debomy, L.; Courthaudon, J. L.; Miclo, L.; Humbert, G.; Gaillard, J. L. Viscoelastic properties of oil-water interfaces covered by bovine β-casein tryptic peptides. *J. Dairy Sci.* **2000**, *83* (11), 2410–2421.
(30) Briand, L.; Chobert, J. M.; Haertle, T. Tryptic hydrolysis of esterified β-casein and β-lactoglobulin. *Milchwissenschaft* **1994**, *49* (7), 367–371.
(31) Yun, S. E.; Ohmiya, K.; Shimizu, S. Role of the phosphoryl group of β-casein in milk curdling. *Agric. Biol. Chem.* **1982**, *46* (6), 1505–1511.
(32) Caessens, P.; Gruppen, H.; Visser, S.; vanAken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997**, *45*, 2935–2941.
(33) Scollard, P. G.; Beresford, T. P.; Murphy, P. M.; Kelly, A. L. Barostability of milk plasmin activity. *Lait* **2000**, *80* (6), 609–619.

Received October 23, 2008. Revised Manuscript Received January 13, 2009. M.E.B. is grateful for the financial support of a VENI grant from the technology foundation STW, Applied Science Division of NWO, and the technology program of the Ministry of Economic Affairs.