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Correlations between Biochemical Characteristics and Foam-Forming and -Stabilizing Ability of Whey and Casein Hydrolysates

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Whey protein and casein were hydrolyzed with 11 commercially available enzymes. Foam properties of 44 samples were measured and were related to biochemical properties of the hydrolysates using statistical data analysis. All casein hydrolysates formed high initial foam levels, whereas whey hydrolysates differed in their foam-forming abilities. Regression analysis using the molecular weight distribution of whey hydrolysates as predictors showed that the hydrolysate fraction containing peptides of 3−5 kDa was most strongly related to foam formation. Foam stability of whey hydrolysates and of most casein hydrolysates was inferior to that of the intact proteins. The foam stability of casein hydrolysate foams was correlated to the molecular weight distribution of the hydrolysates; a high proportion of peptides >7 kDa, composed of both intact casein and high molecular weight peptides, was positively related to foam stability.

KEYWORDS: Hydrolysis; milk proteins; foam properties; molecular weight distribution

INTRODUCTION

Proteins are used in all kinds of food products to profit from their nutritional value or from specific functional properties. Their physicochemical behavior might, however, also impede the use of proteins in products, for example, in high-energy drinks, where high viscosity or limited solubility restricts the protein concentration. Protein functionality can be modified by enzymatic hydrolysis, which alters, for instance, solubility, viscosity, emulsion, and foam properties. Choice of enzyme and process conditions influence hydrolysate composition and thereby the functional properties. This paper will focus on the foam properties of whey and casein hydrolysates.

Foam formation and foam stability should be regarded as two separate processes, influenced by different molecular properties (*1*). Foam formation is influenced by the ability of the foaming agents to quickly migrate to and adsorb on the air−water interface and their ability to reduce the surface tension. Flexibility of proteins is an important factor in the reduction of surface tension (*2*). Foam instability is caused by drainage, Ostwald ripening (disproportionation), and coalescence (film rupture). Drainage of liquid from films causes stretching of the air−water interface, which results in thinning of the film and possible film break. The stretching of films can be opposed by forming viscoelastic films via coverage of the film by proteins that interact by attractive forces such as van der Waals forces and hydrophobic interactions. Low molecular weight surfactants stabilize foams against drainage by the so-called Marangoni effect: stretching of the interface results in a local decrease of surfactant concentration, which forces surfactants to move along the film toward the area with high surface tension, resulting in a liquid flow to the thin part of the film (*2*).

Molecular mechanisms influencing foam formation and foam stability in protein foams have been studied for some isolated proteins [for a review, see Damadoran (*2*)]. Foam properties of hydrolysates may differ considerably from those of their parental proteins. On the one hand, hydrolysis of proteins results in a reduction of molecular weight, which might promote foam formation due to the faster diffusion of molecules to the interface (*3*). On the other hand, peptides formed during hydrolysis might destabilize protein foams by displacement of proteins or by disturbing protein−protein interactions (*3*, *4*). Furthermore, hydrolysis leads to increased charge density, which might negatively influence foam stability, because foam stability was shown to improve when electrostatic repulsion of proteins is minimal (*4*−*6*).

Hydrolysates contain a variety of peptides, differing in their functional behaviors. Fractions obtained from a plasmin hydrolysate of β-casein showed clear differences in their foam-stabilizing properties and in their interfacial behaviors; the surface pressure increase was higher for hydrophobic fractions than for amphipathic fractions (*7*). Some fractions from a β-lactoglobulin hydrolysate exhibited improved interfacial adsorption relative to the original hydrolysate and the intact protein. Analysis of the sequence of the peptides in these

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fractions showed that the presence of distinct hydrophilic and hydrophobic areas contributes to good interfacial properties (*8*).

Althouse et al. (*9*) tested the foam-forming ability and foam stability of whey protein hydrolysates produced by five different enzymes. They showed that foam capacities (measured as percent overrun) of hydrolysate permeates, obtained after ultrafiltration over a 10 kDa membrane, were higher than those of the corresponding retentates. Lieske and Konrad (*10*) tested foam properties of papain hydrolysates of whey proteins, with degrees of hydrolysis of ~3%. The foam overrun increased at pH 6−8, and foam stability was higher over the entire pH range. Small peptides seemed to contribute to foam formation and foam stability, because removal of peptides smaller than 1 kDa by ultrafiltration resulted in impaired foam properties. Studies describing foam stability of casein hydrolysates (*11*, *12*) do not consider relationships with the molecular weight distribution (MWD) of hydrolysates. Although few results concerning formation and stability of casein hydrolysate foams are published, several studies have been published on the emulsion properties of casein hydrolysates, which are also of interest as formation and stabilization of foams and emulsions are governed by essentially similar interfacial properties (*1*, *2*). For casein hydrolysates both increased and decreased emulsion-forming abilities were measured with hydrolysates prepared with various enzymes, having different degrees of hydrolysis (*13*−*17*). Emulsion stability generally decreased upon hydrolysis (*13*, *14*, *16*). Emulsion stability toward coalescence was shown to be correlated to a high proportion of peptides larger than 2 kDa (*17*).

Comparison of foam characteristics of protein hydrolysates reported in the literature is difficult because foam characteristics strongly depend on the methods used to prepare foams and methods used to analyze foam formation and stabilization (*3*, *18*, *19*). Moreover, foam properties depend on factors such as pH (*7*) and ionic strength (*4*), which also differ between the various studies.

The aim of this study is to compare foam properties of hydrolysates made with various enzymes in order to improve the current knowledge about factors important for foam properties. For good comparison, variations in experimental conditions have to be excluded. Therefore, in the present study casein and whey protein were hydrolyzed with a large set of commercially available enzymes to various degrees of hydrolysis. Biochemical properties as well as foam-forming ability and foam stability were measured according to standard protocols. The results were analyzed with statistical data analysis to investigate correlations between hydrolysate characteristics and their foaming properties.

MATERIALS AND METHODS

Materials. Sodium caseinate (89% protein based on dry weight) was obtained from DMV International (Veghel, The Netherlands) and whey protein (WPC 60) from Milei GmbH (Stuttgart, Germany). Acetonitrile, tetrahydrofuran, methanol, and Tris were purchased from Biosolve, and TNBS (5% w/v) was from Sigma-Aldrich. Aqua Purificata was obtained from BUFA BV, potassium tetraborate tetrahydrate was from Fluka, and DTT was obtained from ICN. All other chemicals were of analytical grade obtained from Merck. Milli-Q water was prepared with a Millipore system, and water was filtered over a 0.22 μm filter (Millipak).

An HPLC system from Shimadzu was used with system controller (SLC-10A), HPLC pump (LC-10Ai), injector (SIL-10Ai), column oven (CTO-10AC), UV detector (SPD-10Avp), or fluorescence detector (RF-10Axi).

Production of Hydrolysates. Casein and whey protein were hydrolyzed with 11 different enzymes, under conditions as given in **Table 1**, based on optimum hydrolysis conditions as given by the manufacturers. Enzymes were obtained from Novo Nordisk (Pem, Flavourzyme, Alcalase), Biocatalyst (Promod 184, Promod 258, Pepsin), Genencor (Protex 6L), Amano (NewlaseF), Rohm (Corolase PP, Corolase L10), and Valley Research (Validase FP). The enzymes are commercially available enzyme preparations with broad substrate specificity. Protein suspensions or solutions of 800 mL 5% (w/w) protein were hydrolyzed in a pH-Stat setup (Titrino 718, Metrohm). Whey protein suspensions were pretreated for 15 min at 90 °C to improve accessibility of the whey proteins to the proteolytic enzymes (*20*); casein solutions were not pretreated. In preliminary experiments the maximum degree of hydrolysis was determined for each enzyme/substrate combination. Enzyme concentration (adjusted to a concentration sufficient to reach maximum hydrolysis within 3 h of hydrolysis) and sample time in final hydrolysis were based on these preliminary results. Samples (200 mL) were taken at one-third, two-thirds, and the maximum degree of hydrolysis. Enzymes were inactivated by heating for 15 min at 90 °C. A small amount of the total hydrolysate (~2 mL) was taken apart for determination of the degree of hydrolysis. The remaining hydrolysate was centrifuged (30 min, 3000*g*, 20 °C) at the pH of hydrolysis. Supernatant and pellet were both freeze-dried. Supernatants were used for SEC, determination of free amino acid content, SDS-PAGE, and foam experiments.

Table 1. Hydrolysis Conditions

enzyme	pH	temp (°C)	E/S[a] (%) casein (Cn[b])	E/S[a] (%) whey (Wc[b])
pepsin (Pep)[c]	3	50	5	5
Newlase F (Nwf)	3	50	1	4
Validase FP (Vfp)	3	50	5	5
Promod 258 (P58)	5.5/7[d]	45	3	3
Promod 184 (Brm)	6/7[d]	50	1	3
Flavourzyme (Flz)	6/7[d]	50	1	5
Corolase L10 (Cl1)	6.5	60	3	3
Protex 6L (Px6)	8	60	1	3
Alcalase (Alc)	8	60	1	3
Corolase PP (Cpp)	8	50	1	3
Pem (Pem)	8	45	0.5	2

[a] Enzyme to substrate ratio in % w/w. [b] Abbreviation of protein, used in sample codes of hydrolysates. [c] Abbreviation of enzyme, used in sample codes of hydrolysates. [d] Whey protein hydrolysis was performed at pH 7.

The above-described procedure yields 3 hydrolysates of each protein/enzyme combination, resulting in 33 casein and 33 whey protein hydrolysates. Sample codes are subsequently composed of two digits for protein source, three digits representing the enzyme used, and two digits encoding the degree of hydrolysis reached, for example, CnNwf06: casein, Newlase F, DH = 6%. Protein and enzyme codes are given in **Table 1**.

Protein Determination. Protein concentration was measured by determination of total nitrogen on an N-analyzer (NA 2100 Protein, CE instruments). For calculation of protein concentration a Kjeldahl factor of 6.38 was used.

Degree of Hydrolysis. Degree of hydrolysis was measured spectrophotometrically according to the method of Adler-Nissen (*21*), adapted for use in microtiter 96 well plates. Hydrolysate samples (total hydrolysate) were diluted in 1% SDS solution to a concentration of 0.05% (w/v on protein basis), and the starting protein solution was diluted to 0.1% (w/v on protein basis). A leucine concentration range was used as standard curve. Sample solution (15 μL) was mixed with 45 μL of 0.21 M sodium phosphate buffer (pH 8.2) and 45 μL of 0.05% TNBS in a well. The covered well plate was incubated for 1 h in a 50 °C oven. The reaction was stopped by addition of 90 μL of 0.1 M HCL, and absorption at 340 nm was measured with a Packard Spectra Count plate reader.

Apparent Molecular Weight Distribution. The MWD of supernatants was determined by size exclusion chromatography using a TSK-gel G 2000 SWXL (7.8 × 300 mm column, Toso Haas) connected to a TSK-gel SW precolumn (7.5 × 75 mm, Toso Haas). Analysis was performed at 25 °C, with a flow rate of 0.25 mL/min using an injection of 20 μL of a 2 mg/mL protein solution. The mobile phase was

composed of 8% (w/w) acetonitrile and 0.15% (v/w) TFA in 0.15 M sodium biphosphate solution. The column was calibrated with 17 protein/peptide standards: bovine albumin (M_r = 66000), ovalbumin (M_r = 45000), β-lactoglobulin (M_r = 18400), α-lactalbumin (M_r = 14200), cytochrome *c* (M_r = 12327), Ala-Gln (M_r = 217), Ala-Asp (M_r = 204), and Gly-Leu (M_r = 188) from Sigma and aprotinin (M_r = 6500), ACTH (porcine) (M_r = 4567), insulin A-chain (M_r = 2532), angiotensinogen (M_r = 1759), bradykinin (M_r = 1060), Leu-Trp-Met-Arg-Phe-Ala (M_r = 823), $(Cys\text{-}Tyr)_2$ (M_r = 567), Ala-Pro-Tyr-Ala-Ala (M_r = 492), and $(Ala)_4$ (M_r = 302) all from Serva. Hydrolysate samples were dissolved in eluent, and undissolved particles (present in only some whey supernatant samples) were removed by filtration over a 0.45 μm cellulose acetate filter. The eluate was monitored at 200 nm.

The chromatogram was arbitrarily divided in eight fractions, which, on the basis of the calibration curve, corresponded with the following apparent molecular weight ranges: >20, 15−20, 10−15, 7−10, 5−7, 3−5, 1−3, and <1 kDa. The proportion of each fraction was expressed as percent relative to the total area under the chromatogram.

Free Amino Acid Content. Solutions of hydrolysate supernatants (0.02−3.5% w/v on protein basis) were treated with 4% (v/v, final volume) perchloric acid to precipitate peptides and intact protein. Precipitates were removed by filtration over paper filter (Schleicher & Schluell, 595 $^1/_2$). Nonclear filtrates were subsequently filtered over a 0.45 μm cellulose acetate filter. Clear filtrates were diluted 20 times in mineral-free water (Aqua Purificata).

Samples and a standard amino acid mixture (1% v/v, Sigma AA-S-18) were analyzed by precolumn derivatization with OPA reagent [similar to the method of Burbach et al. (*22*)], followed by separation on a reversed phase C18 column [Superspher 100 RP-18(e), 125 × 4 mm] and fluorometric detection (λ_{ex} = 340 nm, λ_{em} = 455 nm). The amino acids proline and cysteine cannot be detected using this method. The elution system consisted of eluent A composed of sodium citrate buffer (0.1 M) containing 0.33% (v/v) nitric acid and 2% (v/v) tetrahydrofuran, adjusted to pH 5.0, and eluent B composed of 54% (w/w) methanol, 19% (w/w) acetonitrile, 2% (w/w) tetrahydrofuran, and 25% (w/w) distilled water. Samples (10 μL) were eluted with the following gradient: 0−23 min from 20 to 80% eluent B, 23−25 min 80% eluent B, 25−26 min to 100% eluent B, followed by 4 min of regeneration with 80% eluent A. The flow rate was 0.7 mL/min, and the column temperature was 30 °C. Both eluents were filtered over a 0.45 μm membrane filter (Schleicher & Schluell, RC 55) before use.

For calculation of amino acid content in samples, peak areas of individual amino acids were calculated and converted to amino acid concentrations using the peak areas of amino acids in the standard solution. The total amino acid content of the samples was calculated by summing individual amino acid concentrations. Free amino acid content was expressed as percent (w/w) relative to total protein in starting hydrolysate.

Gel Electrophoresis. SDS gel electrophoresis of hydrolysate supernatants was performed on a PhastSystem (Pharmacia Biotech) using a PhastGel homogeneous 20. Sample buffer consisted of 10 mM Tris and 1 mM EDTA, adjusted to pH 8.0 with 2 N HCl. Hydrolysates (±10 mg of protein) were dissolved in 1 mL of sample buffer. Subsequently, 145 μL of sample was mixed with 50 μL of 10% (w/v) SDS, 2.5 μL of 1% (w/v) bromophenol blue, and 2 μL of 50% (w/v) DTT. Samples were heated for 5 min at 100 °C and were subsequently treated with 2 μL of 50% (w/v) DTT. For analysis, 4 μL of sample was applied on the gel. The electrophoresis was run according to a separation method of Pharmacia as described in Separation Technique File 111, with shortened separation time (80 V h). Protein bands were stained with Coomassie blue using the method as described in Pharmacia Development Technique File 200. As molecular weight reference Pharmacia LMW electrophoresis kit (Pharmacia Biotech 17-0446-01) was used.

Solubility. Freeze-dried supernatants were weighed, and protein content was determined. Solubility was expressed as the proportion (percent) of protein in supernatant, relative to total protein content of starting material.

Foam-Forming Ability and Stability. Of each protein/enzyme combination two of three hydrolysates were randomly selected for foam measurements. Foam was prepared with 0.05% (w/v) supernatant protein solutions in 0.02 M imidazole/HCl buffer (pH 6.7) containing 0.34% (w/v) NaCl, with a whipping method as described by Caessens et al. (*7*). A volume of 100 mL of hydrolysate solution was placed in a graduated glass cylinder and whipped for 70 s by a rotating propeller at 2500 rpm at 20 °C (**Figure 1**). Measurements were performed in duplicate. The foam-forming ability was defined as initial foam volume (F_0, measured at 2 min after the start of whipping). Foam volume was followed during 1 h. For statistical analysis foam stability was expressed as the percent foam volume remaining after 15 min (Vf_{15}) or after 1 h (Vf_{60}) relative to the initial foam volume.

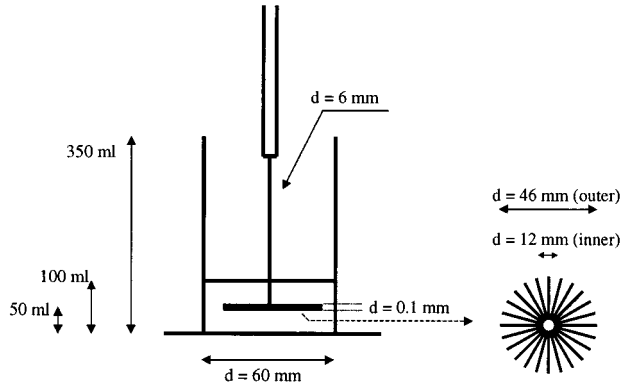

Figure 1. Schematic representation of the experimental setup for the foam formation and stability test with a detail of the small impeller used, d = diameter. Reprinted with permission from ref 7. Copyright 1997 American Chemical Society.

Multivariate Data Analysis. Statistical data analysis was performed using a multivariate data analysis program (The Unscrambler, CAMO). Correlations between sample characteristics were studied by calculation of correlation coefficients and by partial least squares (PLS) regression.

Regression models were made using MW fractions as *x* variables and foam-forming ability or foam stability as the *y* variable. Both *x* and *y* variables were standardized. As the molecular weight fractions are mutually correlated, linear regression analysis will give collinearity problems, and interpretation of regression coefficients is not possible. Therefore, PLS regression, a multivariate regression analysis technique that first decomposes the original data to new independent variables, was used. The PLS regression yields regression coefficients that represent the influence of *x* variables (the MW fractions) on the functional parameters. Moreover, values for the functional parameters are predicted for each sample by use of their MW fractions. For all calculations leave-one-out cross-validation was used as the validation method, which means that predicted values are calculated from a regression model that does not include the predicted sample. The accuracy of the regression model was determined by regarding the correlation between the predicted and measured values for the studied functional property.

Regression analysis was also performed with complete GPC chromatograms as predictors. However, the use of complete chromatograms instead of molecular weight fractions did not result in better predictions for foam properties.

RESULTS AND DISCUSSION

Protein Hydrolysis. The hydrolysates produced with the various enzymes differed considerably in their biochemical properties. **Table 2** shows minimum and maximum values for the solubility and some biochemical properties of 22 casein and 22 whey protein hydrolysates used for foaming experiments. Whey protein and casein were (partly) hydrolyzed by all enzymes, but the final extent of hydrolysis depended on the proteases. The enzyme with lowest activity for both protein sources was Corolase L10, reaching maximum DH values of 5 and 6.5% for casein and whey protein hydrolysis, respectively. In some samples a high amount of peptides/proteins larger than

Table 2. Properties of Casein and Whey Hydrolysates Used for Foam Experiments[a]

	casein	whey
DH (%)	0.5–22	5.5–24
free amino acids[b] (%)	0.04–12	0.09–23
solubility[c] (% protein)	38–85	18–96
apparent MWD[d] (%)		
>20 kDa	0.1–22	0.2–26
15–20 kDa	<0.1–6.5	0.1–4.1
10–15 kDa	<0.1–9.3	0.3–6.6
7–10 kDa	0.2–9.9	0.5–6.4
5–7 kDa	0.5–11	0.6–8.5
3–5 kDa	3.7–20	2.5–17
1–3 kDa	21–49	26–47
<1 kDa	12–51	11–61

[a] Values represent the highest and lowest values measured over all casein or whey samples included in correlation studies. [b] Expressed as % (w/w) of free amino acids relative to total protein. [c] Expressed as % (w/w) protein in supernatant relative to protein in starting material. [d] Expressed as area % relative to total area of size exclusion chromatogram.

20 kDa was present, which most probably is intact protein. The values of the MW fractions given in **Table 2** concern the minimum and maximum values over all samples; the reported values often do not belong the same sample. The high molecular weight fractions calculated from the results obtained with the TSK column are generally slightly higher than those obtained with the Superdex 30 column used in a previous study (*17*). Differences between the two columns were expected because ion and hydrophobic interactions interfere with the separation on hydrodynamic volume (*23*−*25*). These interactions depend on the column material and the eluents used in SEC (*26*, *27*). Nevertheless, the overall trends obtained from the two size exclusion columns are highly comparable.

Free amino acid content was higher in whey hydrolysates than in casein hydrolysates. Only two enzymes showed considerable exo-peptidase activity; free amino acid content in most samples did not exceed 3%.

Foam-Forming Ability. Some typical examples representing the decrease of foam volume with time of casein and whey hydrolysate foams are given in **Figure 2**. The foam volume after 2 min is used as a measure for foam-forming ability.

Whey protein hydrolysates vary in their foam-forming ability (**Figure 2A**) from nonfoaming to foam formation similar to that of intact whey (WPC60). Only one hydrolysate (WcAlc06) resulted in more foam than the intact whey protein (not shown). Five whey protein hydrolysates, all made with different enzymes, did not foam at all.

It might be hypothesized that the peptide concentration (0.05%) used for foaming experiments in our study was too low to increase foam-forming ability. Althouse et al. (*9*) studied whey hydrolysate foams prepared with 5% hydrolysate at pH 7 and found improved foam capacity (percent overrun). The foam-forming ability of whey protein concentrate depends on the protein concentration, with a reported optimum of ∼10% (*28*). However, the foam-forming ability of β-lactoglobulin tested with the same whipping method as used in the present study was rather constant at concentrations ≥0.05% (w/v) (*29*). Moreover, hydrolysates of β-lactoglobulin, also tested with the same method, but at an even lower concentration of 0.01%, formed similar or higher foam levels than the intact protein (*30*). In conclusion, the protein concentration should be sufficiently high to measure improved foam-forming ability.

The different results obtained with the β-lactoglobulin hydrolysates (*30*) and the whey protein concentrates of the present study may be attributed to differences in degree of hydrolysis,

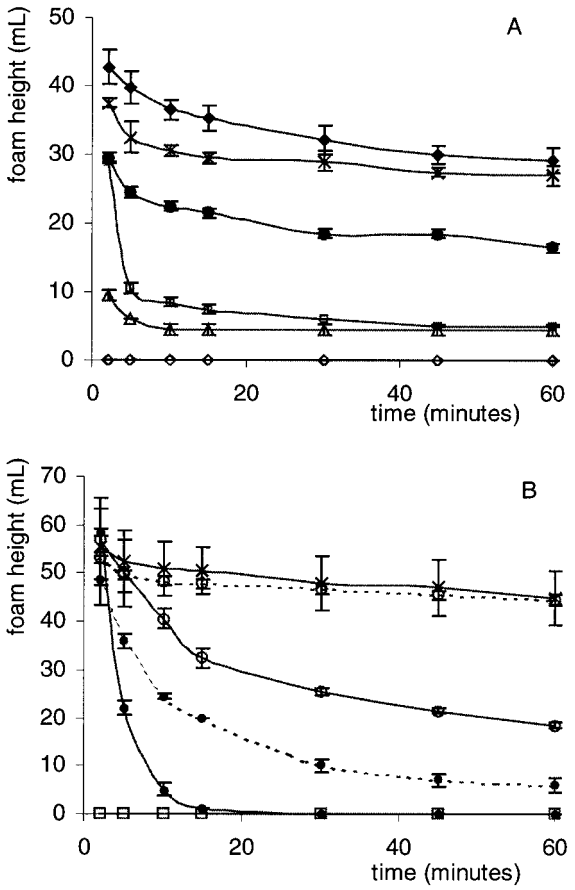

Figure 2. Foam volumes as produced with various whey protein hydrolysate solutions (A) [(◆) WPC60, (×) WcCpp09, (●) WcPem06, (□) WcPx611, (△) WcP5807, (◇) WcVfp06] and casein hydrolysate solutions (B) [(- -○- -) casein, (□) CnAlc19, (●) CnPep06, (- -●- -) CnPx608, (○) CnCl102, (×) CnVfp07] at pH 6.7, as a function of time after whipping (means of duplicate measurements). Error bars are shown. CnAlc19 did form a voluminous foam, but it was highly unstable and all foam disappeared before the first measuring point.

which are 1−4% and >5.5%, respectively, to the use of an isolated protein instead of a whey protein concentrate, or to the use of other enzymes.

Intact casein and casein hydrolysates formed more foam than whey and whey protein hydrolysates (**Figure 2**). All casein hydrolysates formed initial foam volumes comparable to the foam level obtained with intact casein. For three hydrolysates (CnAlc14, CnAlc19, and CnPx618) low foam volumes were measured. However, these samples did form voluminous foam, but the foams were highly unstable, resulting in almost complete disappearance of the foam within the first minute after whipping.

The good foam forming ability of casein is also measured by others (*31*, *32*) and can be attributed to the high flexibility of the protein. A casein hydrolysate with a high degree of hydrolysis prepared with Alcalase showed a significant decrease in foam capacity (percent overrun) compared to intact protein (*33*), whereas in another study foam levels of Alcalase and papain casein hydrolysates were comparable to those of intact casein. Protein concentration, pH, and degree of hydrolysis were not mentioned in this study (*12*). A study concerning the functional properties of hydrolysates from casein showed that 11 of 15 enzymes were able to produce hydrolysates that improved foam capacity by >50%. Unfortunately, details about hydrolysis conditions and biochemical properties of the hydrolysates were not given (*11*). Foam-forming ability and foam

Table 3. Grouping of Whey and Casein Hydrolysates According to Their Foam Stability and for Group WIII According to Their Foam-Forming Ability

	Group	Foam stability[a]	Foam formation[b]	DH range	Enzymes[c]
Whey	WI	+++	High	10	Wc***Cpp***09
	WII	++-	High	6-12	Wc***Cpp***12, Wc***Pem***06, Wc***Pem***10, Wc***Brm***06, Wc***Pep***05
	WIII a	---	High	6-11	Wc***Brm***05, Wc***Alc***06, Wc***Cl1***07, Wc***Nwf***07, Wc***Px6***11
	b	---	Low	6-10,15	Wc***Cl1***06, Wc***Pep***10, Wc***Flz***09, Wc***P58***07, Wc***P58***15, Wc***Vfp***07
	c	---	None	7-24	Wc***Alc***23, Wc***Px6***17, Wc***Nwf***14, Wc***Flz***24, Wc***Vfp***06
Casein	CI	+++	High	4-7	Cn***Vfp***04, Cn***Vfp***07, Cn***Nwf***06
	CII	++-/+--	High	0.5-13,18	Cn***Nwf***10, Cn***Cpp***11, Cn***Cpp***18, Cn***Brm***01, Cn***Pep***02, Cn***Flz***01, Cn***Cl1***02, Cn***Cl1***05, Cn***Pem***09, Cn***Pem***13
	CIII	---	High	6-22	Cn***Brm***06, Cn***Flz***15, Cn***Pep***06, Cn***Alc***14, Cn***Alc***19, Cn***P58***15, Cn***P58***22, Cn***Px6***08, Cn***Px6***18

[a] Criteria for foam stability are based on foam remaining after 1 h, Vf_{60}/Vf_0 values: +++, >70%; ++–, 40–70%; +– –, 10–40%, – – –, < 10%. [b] High initial foam: foam volume > 25 mL. Low initial foam: 5–20 mL (**Figure 1**). [c] Enzyme codes are bold italic; for abbreviations see **Table 1**.

stability of sodium caseinate hydrolysates made with a *Bacillus* proteinase measured at pH 8 were better than that of the intact protein, as long as the degree of hydrolysis did not exceed 3% (*34*).

It was shown that all casein hydrolysates are able to form high initial foam levels, whereas the foam-forming ability of whey hydrolysates does differ between the samples. Foam formation is governed by three factors: transportation, penetration, and reorganization of the molecules on the air−water interface. These processes depend on the size, surface hydrophobicity, and structural flexibility of the surfactants (*3*). The variation in DH and molecular weight distribution between the whey and the casein hydrolysates is comparable (**Table 2**), so the difference in molecular weight distribution or DH cannot explain the contrasting behavior. For adsorption on the air−water interface molecules should contain hydrophobic regions (*8*, *35*). In whey proteins hydrophobic and hydrophilic amino acids are distributed quite uniformly over the entire protein, whereas casein proteins contain distinct hydrophobic and hydrophilic domains (*28*). Therefore, casein hydrolysates are more likely to contain amphiphilic peptides than whey protein hydrolysates, which probably explains their superior foam-forming ability.

Foam Stability. Only one hydrolysate of the 22 tested whey hydrolysate samples, that is, WcCpp09, resulted in a foam that was nearly as stable as the foam made with intact whey (**Figure 2A**). All other whey hydrolysates formed foams with considerably lower stability. The hydrolysates could be tentatively grouped according to the difference in their ability to stabilize foams (**Table 3**). The first group of hydrolysates forming stable foams (WI) consists only of WcCpp09. The second group of hydrolysates (WII), consisting of five samples, formed moderately stable foams, with final foam volumes of ∼17 mL and a maximum foam volume decrease of 60% (remaining stability minimal 40%). The decrease of foam volume in time and final foam volume was very similar for the various samples within this group, showing curves represented by sample WcPem06 in **Figure 2A**. The hydrolysates constituting the third group (WIII) formed foams that collapsed entirely within the observation period of 60 min; these foams were denoted unstable. In some cases little foam was left sticking to the glass cylinder, but in the middle all foam was gone. Most of the foam instability was observed during the first 15 min. Samples belonging to this group differed in their ability to form foams; some hydrolysates did not form any foam at all, whereas others reached levels similar to that of intact whey. Hydrolysates of similar DH values as well as hydrolysates made with one enzyme belong to different groups (**Table 3**), which indicates that enzyme specificity and DH cannot explain differences in foam stability in the case of whey hydrolysates.

In the literature both increased and decreased foam stabilities of whey hydrolysate foams are reported (*10*, *36*, *37*).

All casein hydrolysates were able to form foams with high initial foam levels, but the stability of the foams varied considerably (**Figure 2B**). According to differences in stability the casein hydrolysates could be divided tentatively into three groups (**Table 3**). One group of hydrolysates was able to stabilize the hydrolysates very well, and the foam stability was comparable to that of intact casein foam. Three hydrolysates belong to this group (CnVfp04, CnVfp07, and CnNwf06). The second group consists of hydrolysates that stabilized the foams to some extent; the remaining foam volume over 1 h varied from 10 to 70%. The third group includes nonstable foams; all foam disappeared within 1 h. As with foams made with whey hydrolysates, some foams of this group stuck to the glass wall at the end of the observation period. Casein hydrolysates made with the same enzyme belonged to the same group in the case of 7 of the 11 enzymes used. This might point to the fact that the enzyme specificity influences the stability of casein hydrolysate stabilized foams, in contradiction to the absence of enzyme influence in the case of the whey hydrolysates.

In many casein foams destabilization by disproportionation and coalescence of air bubbles was observed. Most samples formed foams with small air bubbles, but after ∼5 min larger bubbles appeared. Small air bubbles dissolved in larger neighboring bubbles or two bubbles melted together, resulting in

Table 4. Correlation between Biochemical Properties and Foaming Properties of Hydrolysates

	whey protein hydrolysates			casein hydrolysates		
	F_0[a]	Vf_{15}[b]	Vf_{60}[c]	F_0	Vf_{15}	Vf_{60}
DH	–0.77	–0.03	–0.26	–0.54	–0.53	–0.53
apparent MWD						
>20 kDa	0.62	0.12	0.22	0.38	0.64	0.73
15–20 kDa	0.76	0.23	0.42	0.43	0.73	0.79
10-15 kDa	0.78	0.16	0.37	0.51	0.74	0.78
7–10 kDa	0.81	0.17	0.36	0.52	0.68	0.66
5–7 kDa	0.79	0.15	0.35	0.48	0.57	0.55
3–5 kDa	0.85	0.13	0.24	0.47	0.48	0.43
1–3 kDa	–0.47	0.12	0.29	–0.49	–0.71	–0.76
<1 kDa	–0.75	–0.23	- 0.24	–0.52	–0.65	–0.64

[a] F_0 = initial foam volume. [b] Vf_{15} = % foam volume remaining after 15 min (in % relative to foam volume at $t = 0$). [c] Vf_{60} = % foam volume remaining after 60 min (in % relative to foam volume at $t = 0$).

larger bubbles that finally broke. In the case of whey hydrolysates this was also observed, but in fewer samples than with casein hydrolysates.

Destabilization of foams is often measured by recording the liquid that drains from the foam, rather than by observing the foam and recording the decrease of foam volume. This might explain the fact that little is published about destabilization mechanisms in protein hydrolysate stabilized foams. Caessens et al. (*30*) investigated the stability of some foams prepared with β-lactoglobulin hydrolysates and reported that coalescence did not appear during the observation time.

The observed foam properties of hydrolysates are a result of both protein breakdown and conformational changes induced by heat treatment and pH adjustments. The effects of individual processing steps were not considered separately, because the study was aimed at comparing final hydrolysate products. Enzymes constitute 0.5−5% of the total protein content. After hydrolysis, the enzymes are denatured and probably discarded with centrifugation. Hydrolysates from one enzyme differ in foam properties (**Table 3**), which indicates that if enzymes are not completely removed, they do not significantly influence foam properties.

Correlation of Foam Properties with Other Hydrolysate Characteristics. To investigate the factors influencing the foam-forming ability and foam-stabilizing abilities of whey and casein hydrolysates, correlation coefficients between the foam properties and other hydrolysate characteristics were calculated (**Table 4**). The correlation coefficients for foam-forming ability of casein hydrolysates were calculated by excluding the highly unstable samples, because their foam had already collapsed before the first measuring point.

Correlation between Degree of Hydrolysis and Foam-Forming Ability and Foam Stability. From **Table 4** it can be seen that some correlation exists between the degree of hydrolysis and the foam formation of whey and casein hydrolysates.

Concerning foam stability of casein hydrolysates, a general trend is observed showing a decrease of foam stability with increasing DH. Six of the nine casein hydrolysates forming nonstable foams (group CIII) had a DH >14%. From the 10 hydrolysates forming intermediately stable foams (group CII) only one hydrolysate had a DH >14%. Therefore, it can be presumed that high-DH hydrolysates are generally unable to form stable foams. The other hydrolysates vary in their foam stability, independently of the DH value.

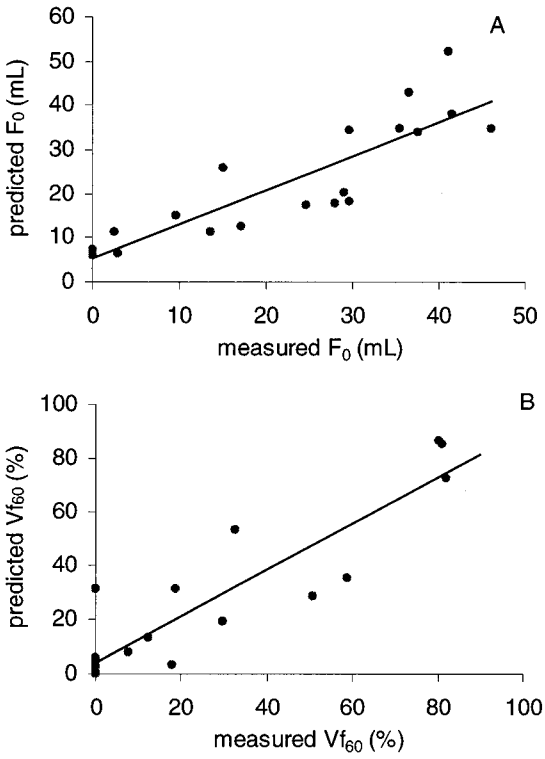

Figure 3. Correlation between predicted and measured values for (A) initial foam volume (F_0) of whey hydrolysate foams ($r = 0.86$) and (B) remaining foam volume (Vf_{60}) values of casein hydrolysates ($r = 0.90$). Prediction was based on multivariate regression analysis (PLS) with molecular weight fractions of the hydrolysates determined from SEC results as *x* variables.

All whey hydrolysates used for foaming experiments had a DH of 6% or higher. These hydrolysates did not show improved foaming properties compared to intact protein. Most whey protein hydrolysates with high DH (DH >14%) did not form foams at all. For the hydrolysates with a lower DH no clear correlation between DH and foam formation was found. The stability of whey protein hydrolysate foams was not related to the DH of the hydrolysates.

Correlation between Molecular Weight Distribution and Foam-Forming Ability. As shown above, all casein hydrolysates were able to form high initial foams. Therefore, the foam-forming ability does not seem to depend on the MWD of the peptides. Whey hydrolysates, however, did show differences in their foam-forming ability. Calculation of the correlation coefficients between the MWD fractions and F_0 showed that especially the fractions with apparent MW >3 kDa were positively related to foam-forming ability (**Table 4**).

Multivariate regression analysis was used to study the relationship between MW fractions and foam-forming ability of whey hydrolysates in more detail. A rather good correlation was found between the initial foam volume as predicted from MW fractions and the measured initial foam volume (**Figure 3A**); the correlation coefficient was 0.86. Study of the regression coefficients showed that the fraction peptides with apparent molecular weight between 3 and 5 kDa had the highest positive regression coefficient. The regression coefficient of the fraction >20 kDa had a negative sign, which means that an increase in this fraction results in a decrease of initial foam. This seems to contradict the positive regression coefficient given in **Table 4**. However, coefficients in **Table 4** are calculated using univariate regression, whereas for regression analysis all molecular weight

fractions, which are interdependent, are used. As the proportion of the fractions are interdependent, the regression coefficients may have a sign different from that of the correlation coefficients. The correlation coefficient between the fraction 3−5 kDa and F_0 was 0.85 (**Table 4**), indicating that the proportion of this fraction can predict the foam forming almost as accurately as the entire MWD. Study of the proportion of this fraction in the hydrolysates showed that, if the fraction 3−5 kDa represented >10% of all peptides, generally >25 mL of foam was formed (data not shown). Remarkably, the fraction >20 kDa does not contribute to the foam-forming ability of hydrolysates, whereas intact whey is able to form a voluminous foam. The fraction >20 kDa contains remaining intact protein and/or peptide aggregates, which cannot be distinguished from each other with the chromatographic technique used. Probably, these high MW compounds are present in too low concentrations or they are less effective in foam forming than the intact whey protein. As the high MW fraction does not contribute to foam-forming properties, the composition was not further investigated.

It should be noted that for determination of the MWD some whey hydrolysate supernatant solutions were filtered over a 0.45 μm filter because small amounts of undissolved particles were observed. On the contrary, in the whey hydrolysate supernatant solutions prepared for the foam experiments no undissolved particles were observed by eye, possibly because of the lower protein concentration. Moreover, if any aggregates would have been present in the hydrolysate solution used to test foam properties, their influence on foam-forming ability was probably of minor importance, because especially the lower molecular weight fractions are responsible for the difference in foam-forming ability.

Althouse et al. (*9*) performed foam studies with ultrafiltrated whey hydrolysates and found that peptides <10 kDa are needed for foam formation, which is in agreement with our findings.

From the presented results concerning the foam-forming ability of whey and casein hydrolysates it might be hypothesized that the first prerequisite for foam-forming ability of hydrolysates is the presence of amphiphilic peptides. If a protein source contains distinct hydrophobic regions, the chance of the presence of these peptides in hydrolysates is high, irrespective of the molecular weight of the peptides formed. However, if the parental protein does not contain these regions, as is the case with whey protein, the MWD of the peptides is an important factor in foam formation. Probably, peptides with a molecular weight >3 kDa are needed to obtain amphiphilic peptides from whey protein. The fact that peptides smaller than ∼10−15 kDa are favored over larger peptides might be explained by the faster diffusion of low molecular weight peptides and possibly the higher flexibility of these peptides.

Correlation between Molecular Weight Distribution and Foam Stability. The correlation coefficients between molecular weight fractions and foam stability of whey hydrolysate foams were rather low (**Table 4**). As was shown above, the whey hydrolysate foams can be grouped according to differences in final foam volume (**Table 3**). However, the differences in foam stability within each group were low, and the MWD of the samples does not correlate to these rather small differences. Foam stability of casein hydrolysates is related to MWD, as shown by the rather high correlation coefficients (**Table 4**). Fractions with positive correlation coefficients contribute to stabilization of the foam, because high proportions of these fractions result in high remaining foam volumes. A high proportion of large peptides, especially >10 kDa, positively influences foam stability. Multivariate regression analysis of MWD fractions and foam stability (Vf_{60}) of casein hydrolysate foams confirmed the correlation between these parameters. In **Figure 3B** the result of the prediction is plotted; the correlation coefficient between measured and predicted values was 0.90.

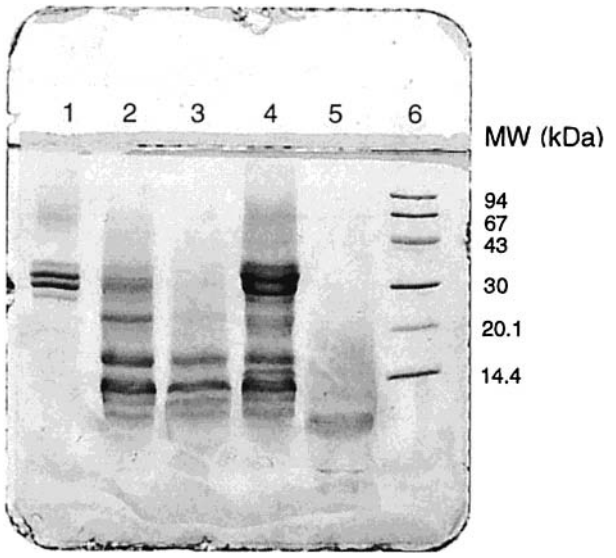

Figure 4. Determination of the presence of intact casein in casein hydrolysates using SDS-PAGE: (lane 1) sodium caseinate; (lanes 2–5) casein hydrolysates CnVfp04 (2), CnVfp07 (3), CnNwf06 (4), and CnCpp11 (5); (lane 6) molecular weight marker.

The regression coefficients (data not shown) had positive values for the fractions with MW >7 kDa. The hydrolysates forming the most stable foams contained >25% peptides >10 kDa. To investigate whether the foam stability of these samples arises from the presence of intact casein, the samples were analyzed with SDS gel electrophoresis to study the composition of the high molecular weight fraction (**Figure 4**). The analysis showed that sample CnNwf06 contained a relatively high amount of intact casein, but high molecular weight peptides were also present. The hydrolysate made with Validase FP with DH = 4% contained some intact casein, whereas in the DH = 7% hydrolysate no intact casein was detected. Both hydrolysates contained high molecular weight peptides. These results show that most probably the foam stability of these hydrolysates is not (only) due to the presence of intact casein, high molecular weight peptides also contribute to the stabilization of the foam.

In the present research, the protein part of hydrolysates was studied in relation with foam properties of hydrolysate solutions. These solutions contain besides protein also fat, minerals, and lactose. In studies concerning foam and other functional properties of whey protein concentrates and isolates it was shown that these components influence the functional behavior of whey protein (*4*, *38*). The mineral concentration in hydrolysates is not expected to influence results of foam experiments in the present study, because foam properties were determined in a buffer containing ions in a concentration that is high enough to eliminate effects of salts in the samples. Fat and lactose are present in low amounts in caseinate, but whey protein concentrate 60 contains 24 and 5% of these components, respectively. The ratio between protein and other components in hydrolysates may differ, because the amount of soluble protein differs between the samples. However, a correlation between the amount of nonprotein material present in foam experiments and foam properties was not found (data not shown), indicating that differences in fat and lactose contents are of minor importance. Another influential factor, not studied in the present research, may come from covalent or non-covalent interactions between

lactose or fat with peptides, resulting in, for example, formation of lactosylated peptides.

In conclusion, it was shown that foam formation by casein hydrolysates is independent of the molecular weight distribution of the peptides, whereas whey hydrolysates should contain a sufficient amount of peptides >3 kDa. Foam stability of casein hydrolysate foams is correlated to the MWD, especially to the fraction peptides with MW >7 kDa. For foam formation the presence of amphiphilic peptides might be the most important factor, whereas for foam stability the presence of relatively high molecular weight peptides seems to be crucial.

ABBREVIATIONS USED

DH, degree of hydrolysis; MWD, molecular weight distribution; TNBS, trinitrobenzenesulfonic acid.

Supporting Information Available: Biochemical properties and foam-forming and foam stability values for whey and casein hydrolysates included in the correlation studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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