Emulsion Properties of Casein and Whey Protein Hydrolysates and the Relation with Other Hydrolysate Characteristics

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Casein and whey protein were hydrolyzed using 11 different commercially available enzyme preparations. Emulsion-forming ability and emulsion stability of the digests were measured as well as biochemical properties with the objective to study the relations between hydrolysate characteristics and emulsion properties. All whey protein hydrolysates formed emulsions with bimodal droplet size distributions, signifying poor emulsion-forming ability. Emulsion-forming ability of some casein hydrolysates was comparable to that of intact casein. Emulsion instability was caused by creaming and coalescence. Creaming occurred mainly in whey hydrolysate emulsions and in casein hydrolysate emulsions containing large emulsion droplets. Coalescence was dominant in casein emulsions with a broad particle size distribution. Emulsion instability due to coalescence was related to apparent molecular weight distribution of hydrolysates; a relative high amount of peptides larger than 2 kDa positively influences emulsion stability.

Keywords: *Hydrolysis; milk proteins; emulsions; degree of hydrolysis; molecular weight distribution*

INTRODUCTION

Proteins are nutritionally important as a source of nitrogen and essential amino acids. Consumption of intact proteins, however, might cause allergic reactions in sensitive individuals (*1*). As an alternative, extensively hydrolyzed proteins can be used in the diet as nutritional value is preserved (*2, 3*). Hydrolyzed proteins might also be beneficial for patients suffering from specific digestion disorders such as cystic fibrosis or short bowel syndrome (*4*) or can be used in high protein diets in case of malnutrition (*5*−*7*).

Enzymatic hydrolysis of proteins does not only affect digestibility and allergenicity of proteins but also induces modification of functional properties such as solubility, viscosity, gelation, and emulsifying and foaming properties (*8, 9*). Hydrolysis of proteins causes changes such as an increase in the number of charged groups, a decrease in the average molecular weight, and exposure of reactive groups, factors that influence emulsion-forming and emulsion-stabilizing abilities of protein hydrolysates (*10, 11*).

Emulsion-forming ability and emulsion stability should be considered as two separate processes that are influenced by other properties of the proteins or peptides used in emulsions (*12*). During the formation of an oil in water emulsion in a homogenizer, surfactant molecules adsorb on the interface of droplets, droplets break up into smaller droplets, and newly formed droplets collide, possibly resulting in recoalescence. The final droplet size is an equilibrium between droplet breakup and droplet recoalescence. Surfactants (e.g., protein or peptides) contribute to the formation of droplets by lowering the interfacial tension (facilitating droplet breakup) and by prevention of recoalescence (*13*). Once emulsions are formed, they are subject to several forms of instability. The three main factors are creaming (primarily depending on droplet size), aggregation, and coalescence. Emulsion stability is favored by proteins and peptides able to oppose attraction between emulsion droplets by promoting electrostatic repulsion or steric hindrance (*14, 15*).

Protein hydrolysate characteristics that are often related to emulsion properties are the degree of hydrolysis (DH) and the apparent molecular weight distribution (MWD). Several authors reported improved emulsion-forming abilities for low DH casein hydrolysates (*8, 16*) and low DH whey protein hydrolysates (*16, 17*) in comparison to the parental materials. On the other hand, reduction of emulsion-forming ability after hydrolysis was also reported for casein hydrolysates emulsions (*18, 19*) and emulsions of whey hydrolysates (*20*). The emulsion stability generally decreases with hydrolysis for all milk proteins and also for low DH hydrolysates (*8, 16, 21*). Regarding the molecular weight of peptides in hydrolysates, a minimum peptide length seems to be desired for good emulsion properties. Peptide mixtures obtained by filtration of whey protein hydrolysates over a 10-kDa membrane showed poor emulsion-forming abilities as compared to the retentates and the parental hydrolysates (*22*). Chobert and coworkers (*8*) found that for tryptic whey hydrolysates good emulsion stability is reached with an apparent molecular weight of at least 5000 Da, while Singh and Dalgleish (*23*) reported that a peptide length of only 500 Da is needed for emulsion stabilization. It is commonly thought that peptide size is not the only factor influencing the emulsion behavior of peptides. As was shown by several authors, amphiphilicity of peptides is important for interfacial and emulsifying properties of pep-

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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 (P6L)	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] E/S = 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.

tides (*24*−*26*). Rahali and co-workers (*26*) analyzed amino acid sequences of β-lactoglobulin peptides adsorbed at an oil/water interface and concluded that amphiphilic character was more important than peptide length for emulsion properties.

As outlined above, the literature is ambiguous about relationships between biochemical and emulsion properties. The hydrolysates used in several studies concerning these relations were produced with various enzyme/substrate combinations, and their functional and biochemical properties were characterized by different methods, which might explain the contradictory results. The best approach to compare functional properties of protein hydrolysates prepared with various enzymes is to produce the hydrolysates and emulsions in a standardized manner and to characterize all hydrolysis products with the same methods. Additionally, the data obtained from this approach can be statistically analyzed, resulting in a more correct definition of the biochemical characteristics that influence functional properties.

Therefore, in the present study, whey protein concentrate and sodium caseinate were hydrolyzed with 11 different commercially available enzymes to various degrees of hydrolysis to study hydrolysates that are similar to commercially available products. All hydrolysates were characterized according to standard protocols. The results were analyzed with statistical analysis to investigate correlations between biochemical properties and emulsion characteristics.

MATERIALS AND METHODS

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

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

Production of Hydrolysates. Casein and whey protein concentrate were hydrolyzed with 11 different enzymes, under the conditions as given in Table 1, based on optimum hydrolysis conditions as given by the manufacturers. Enzymes were obtained from Novo Nordisk (Pem, Flavourzyme, and Alcalase), Biocatalyst (Promod 184, Promod 258, and pepsin), Genencor (Protex 6L), Amano (Newlase F), Rohm (Corolase PP and Corolase L10), and Valley Research (Validase FP). Protein suspensions or solutions of 800 mL 5% (w/w) protein were hydrolyzed in pH-stat setup (Titrino 718, Metrohm). Whey protein suspensions were held at 90 °C for 15 min prior to enzyme digestion; 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 15 min at 90 °C. The hydrolysate was centrifuged (30 min, 3000*g*, 20 °C) at the pH of hydrolysis. Supernatant and pellet were both freeze-dried. 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 means casein, Newlase F, and 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. The DH was measured spectrophotometrically according to the method of Adler-Nissen (*27*), which was adapted for use in microtiter 96-wells plate. Hydrolysate samples were diluted in 1% SDS to a concentration of 0.05% (w/v on protein basis); starting material (protein solutions) was diluted to 0.1% (w/v on protein basis). A leucine concentration range was used as the standard. 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 stove. The reaction was stopped by addition of 90 μL of 0.1 M HCl; absorption at 340 nm was measured with a Packard Spectra Count plate reader.

Apparent Molecular Weight Distribution. The apparent molecular weight distribution (MWD) of supernatants was determined by size-exclusion chromatography, performed with a Superdex Peptide PE 7.5/300 column (Pharmacia) at 30 °C, with a flow rate of 0.5 mL/min using an injection of 20 μL of a 2 mg/mL protein solution. The mobile phase was composed of 30% acetonitrile with 0.15% TFA in Milli-Q water. The column was calibrated with 13 peptide standards: 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; undissolved particles 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 seven fractions, which, on the basis of the calibration curve, corresponded with the following apparent molecular weight ranges: >5 kDa, 4−5 kDa, 3−4 kDa, 2−3 kDa, 1−2 kDa, 0.5−1 kDa, <0.5 kDa. The proportion of each fraction was expressed as percentage 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 concentration) perchloric acid to precipitate the 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 Burbach et al., *28*), followed by separation on reverse-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 are not detected using this method. The elution system consisted of eluent A, composed of sodium citrate buffer (0.1 M) containing 0.33% nitric acid and 2% tetrahydrofuran, adjusted to pH 5.0, and eluent B, composed of 54% methanol, 19% acetonitrile, 2% tetrahydrofuran, and 25% (w/w) distilled water. Samples (10 μL) were eluted with 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 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 & Schuell, RC 55) before use.

For calculation of amino acid content in samples, peak areas of individual amino acid were calculated and converted to amino acid concentrations using the peak areas of amino acids in the standard solution. 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 the hydrolysate.

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

Emulsion Forming and Stability. Of each protein/enzyme combination, two hydrolysates were randomly selected for emulsion measurements. Emulsion properties were measured with a solution of 0.56% (w/v) supernatant protein in 0.02 M imidazole/HCl buffer, pH 6.7, containing 3.44 g/L NaCl and 0.2 g/L NaN_3, according to methods used by Caessens et al. (*29*). A total of 2 mL of tricaprylin oil (Sigma) was added to 18 mL of hydrolysate solution and was mixed by hand-shaking during 40 s to obtain a pre-emulsion. The pre-emulsion was subsequently homogenized in a laboratory-scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands) for 9 passages at 60 bar.

Emulsion-forming ability was investigated by measuring particle size distribution directly after homogenizing with a Malvern Mastersizer (Malvern Instruments, Mastersizer S long-bed version 2.1). Particle size distribution was measured in deionized water as dispersant, using a polydisperse model and presentation code of 3NAD (i.e., refractive index and absorption of emulsion particles of 1.456 and 0, respectively). For statistical analyses, the particle size distributions curves were used as well as the d_{32} values (the volume−surface average particle diameter) as described under Multivariate Data Analysis.

The emulsion stability was determined according to the method described by Pearce and Kinsella (*30*) by measuring the turbidity at 500 nm of samples diluted 100 times in 0.1% SDS at $t = 0$, 1, 3, and 24 h. Samples, diluted 10 times, were checked for remaining aggregates using a light microscope equipped with a camera (Olympus, BH-2) at a magnification of 400×. Emulsion stability (E_{stab}) was expressed as the percentage of emulsion turbidity remaining after 24 h. Moreover, emulsions were judged visually for formation of cream layer and separation of oil.

Multivariate Data Analysis. Statistical data analysis was performed using a multivariate data analysis program (Unscrambler, CAMO). Correlations between sample characteristics were studied by calculation of correlation coefficients and by partial least squares (PLS) regression. To study the correlation between emulsion forming and emulsion stability, normalized (mean normalization) particle size distribution curves were used as *x*-variables, and emulsion stability after 24 h was used as *y*-variable. For other regression analyses, apparent molecular weight fractions (weight: 1/SD) were used as *x*-variables. For all calculations, full cross validation was used as validation method.

RESULTS AND DISCUSSION

General Hydrolysate Characteristics. The 22 casein and 22 whey protein hydrolysates differed strongly in their biochemical properties (Table 2). The degree of hydrolysis varied considerably from 0.5 to 24%; the extent of hydrolysis reached with whey protein was comparable to that of casein. The ranges of the apparent MWD demonstrate that some hydrolysates contained a large amount of high molecular weight peptides. As all molecules larger than ca. 6 kDa elute in the void volume of the size-exclusion column, no information about the presence of intact protein can be obtained from these analyses. With some enzymes, extensive hydrolysis was reached, resulting in hydrolysates containing up to 35% peptides smaller than 500 Da. Free amino acid content ranged between 0.4 and 23%. However, only Flavourzyme and corolase PP have significant exo-protease activity. The other enzymes released low amounts of free amino acids; the maximum concentration did not exceed 3%.

Table 2, Properties of Casein and Whey Hydrolysates Used for Emulsion Experiments

	casein	whey
DH (%)	0.5−22	5.5−24
free amino acids (%)[a]	0.4−12	0.9−23
solubility (%)[b]	38−85	18−96
apparent MWD (%)[c]		
>5 kDa	0.2−75	1.0−63
4−5 kDa	0.8−20	0.7−14
3−4 kDa	2.0−24	1.9−20
2−3 kDa	4.6−24	5.0−20
1−2 kDa	3.2−33	5.1−34
0.5−1 kDa	1.3−32	2.2−31
<0.5 kDa	0.6−30	3.1−35

[a] Expressed as % (w/w) of free amino acids relative to total protein. [b] Expressed as % (w/w) protein in supernatant relative to protein in starting material. [c] Expressed as area % relative to total area of size exclusion chromatogram.

Solubility of intact casein protein is high at neutral pH, but around its iso-electric point (ca. pH 5), it precipitates. Hydrolysis in this pH range resulted in partial resolubilization of the protein. However, solubility did not reach values as high as with hydrolysis at alkaline pH. The whey protein concentrate is not completely soluble in water (pH of WPC in demineralized water is 6.8); a concentration of 5% (w/v) yields a suspension instead of a solution. Hydrolysis at alkaline pH resulted in an increase of solubility to nearly 100%.

Emulsion-Forming Ability. To examine the emulsion-forming abilities of the protein hydrolysates, the emulsion droplets were studied by measuring the particle size distribution immediately after homogenization. Particle size distributions can be summarized using various average particle diameters (e.g., the d_{32} value, the volume−surface average diameter, and the d_{43} value, the weight mean diameter; Table 3). The large emulsion droplets weigh more strongly in calculation of the d_{43} value than in calculation of the d_{32} value. Therefore, emulsions with similar d_{32} that differ in d_{43} (e.g., CnBrm06 vs CnPx618) differ in the amount of large droplets.

The emulsion formed with intact casein consisted of small emulsion droplets; the d_{32} value of the particle size distribution was 0.92 μm. Comparison of all casein hydrolysate emulsions showed the existence of three types of emulsions (Figure 1). Five hydrolysates (group I) formed emulsions comparable to the emulsion made with intact casein, with low d_{32} values (0.56−0.95 μm) and relatively narrow particle size distributions (d_{43} ranges from 1.1 to 2.2 μm). Seven hydrolysates (group II) formed emulsions with broad particle size distributions, containing both small and large emulsion drop-

Table 3. Emulsion-Forming Ability and Emulsion Stability of Casein Hydrolysate and Whey Hydrolysate Emulsions

	emulsion forming		stability		emulsion forming		stability
sample	d_{32} (μm)[a]	d_{43} (μm)[b]	E_{stab}[c] (%)	sample	d_{32} (μm)	d_{43} (μm)	E_{stab} (%)
casein	0.92	1.4	96	whey	0.92	5.4	96
CnAlc14	0.82	13.2	5	WcAlc06	0.63	1.7	88
CnAlc19	0.78	12.9	10	WcAlc23	3.5	13.6	56
CnBrm01	0.65	1.3	98	WcBrm05	0.92	5.5	93
CnBrm06	0.95	1.7	78	WcBrm06	2.7	14.7	93
CnC1002	0.56	1.1	96	WcCl106	2.6	19.4	89
CnC1005	0.85	2.2	78	WcCl107	2.2	17.3	95
CnCpp11	0.81	5.9	45	WcCpp09	1.4	9.6	86
CnCpp18	0.50	4.3	68	WcCpp12	1.1	3.3	95
CnFlz01	0.84	2.0	86	WcFlz09	2.8	15.6	79
CnFlz15	7.4	34.5	36	WcFlz24	1.7	11.6	63
CnNwf06	3.5	40.4	96	WcNwf07	4.6	13.3	67
CnNwf10	4.6	53.5	80	WcNwf14	5.3	12.8	61
CnP5815	7.2	72.2	19	WcP5807	0.65	6.8	78
CnP5822	13.3	70.0	17	WcP5815	2.3	9.6	75
CnPem09	3.6	28.4	97	WcPem06	1.3	4.7	93
CnPem13	2.0	18.0	91	WcPem10	0.98	4.5	93
CnPep02	1.0	5.5	42	WcPep05	3.4	19.1	100
CnPep06	4.2	24.2	64	WcPep10	4.6	20.3	90
CnPx608	1.1	6.8	18	WcPx611	1.3	3.6	78
CnPx618	0.96	12.8	9	WcPx617	3.1	13.6	61
CnVfp04	3.0	31.7	101	WcVfp06	4.8	12.2	95
CnVfp07	4.3	59.3	99	WcVfp07	3.0	9.5	92

[a] Volume−surface average particle size of the emulsion droplets. [b] Weight mean diameter of the emulsion droplets. [c] % turbidity remaining after 24 h.

lets. The volume−surface average particle sizes of these emulsions are comparable to the values of group I, varying between 0.5 and 1.1 μm, but the d_{43} values are considerably higher, ranging from 4.3 to 13.2 μm. The third group represents emulsions with a bimodal distribution, with a first small peak at d_{32} of $\pm$0.33 μm and a main peak at large emulsion droplet sizes with d_{32} varying between 26 and 30 μm. The d_{32} values based on the entire distribution varied between 2 and 14 μm, and the d_{43} values varied between 18 and 72 μm.

According to Walstra and Smulders (*12*), the ability to reduce surface tension influences the formation of small particles and the ability to resist recoalescence is important for preservation of the small particles. Hydrolysates from all groups form small particles, indicating that probably all hydrolysates contain (some) surface-active peptides. The differences in particle size distribution might arise from differences in the ability to prevent recoalescence or from differences in the concentration of the surface-active peptides. The results clearly show that d_{32} values alone give insufficient information for investigation of emulsion forming ability; emulsions with comparable d_{32} can differ substantially in the emulsion droplet size distribution.

The particle size distribution of the emulsion made with intact whey protein was comparable to that of intact casein, having a d_{32} value of 0.92 μm. Emulsions made with whey protein hydrolysates had bimodal particle size distributions (Figure 1), except sample WcAlc06, which formed an emulsion similar to intact whey and casein. The d_{32} values based on the entire particle size distributions varied between 0.65 and 5.3 μm; the average particle size of the first peak varied from 0.32 to 0.44 μm; and that of the second peak varied from 3.4 to 19 μm. The d_{43} values of the bimodal emulsions varied from 3.3 to 20.3 μm (Table 3).

In the present study, none of the whey hydrolysates showed improved emulsion-forming ability as compared to intact whey, while Lakkis and Vilotta (*2*) measured a decrease in particle sizes and a more uniform distribution of emulsion droplets with whey protein hydrolysates made with pepsin, trypsin, or chymotrypsin. Hydrolysis of β-lactoglobulin (*31*) resulted in hydrolysates that produced emulsions with more uniform droplet size distribution than the intact protein, but with similar d_{32} values.

Emulsion Stability. As was mentioned in the Introduction, the main causes of emulsion instability are creaming, aggregation, and coalescence. When emulsion droplets are aggregated, the effective particle size increases, usually resulting in creaming. Creaming is also observed as a result of poor emulsion forming, i.e., the presence of large emulsion droplets after homogenizing. Aggregation and creaming might promote coalescence due to the increased contact time between oil droplets. Since coalescence is the final stage of emulsion in stability, the present study focuses on this type of instability. Creaming was observed visually but was not quantified.

Creaming was observed in the majority of the whey hydrolysate emulsions and in casein hydrolysate emulsions from group III. As was observed from the particle size distributions, these emulsions contain relative high quantities of large emulsion droplets, which are most probably responsible for the creaming.

Instability due to coalescence was quantified by measurement of the turbidity decrease, a method that is generally used to measure emulsion stability (*8, 19, 32*−*35*). Creamed emulsions were turned carefully several times to disturb the cream layer and to create a homogeneous sample without breaking up the oil droplets. Subsequently, emulsions were diluted in SDS to suppress aggregation. The results obtained from the turbidity measurements after 24 h are given in Table 3. The major decrease in turbidity was observed during the first 1−3 h (data not shown). Turbidity decrease in emulsions made with intact casein and whey was 4% for both proteins. Although the whey hydrolysate emulsions creamed rapidly, the turbidity decrease was relatively low. Therefore, it seems that creaming does not necessarily cause increased coalescence, as was also reported by Smulders (*36*). Some casein hydrolysate emulsions were highly unstable, showing a turbidity decrease of 95%. The emulsion droplets were disrupted since an oil layer on top of the solution was observed.

The observed emulsion 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 since the study was aimed at comparing final hydrolysate products. Enzymes constitute 0.5−5% of the total protein. After hydrolysis, the enzymes are denatured and probably discarded with centrifugation. Hydrolysates from one enzyme differ in emulsion properties, which indicate that if enzymes are not completely removed, they do not significantly influence emulsion properties.

Correlation between Emulsion-Forming Ability and Emulsion Stability. As was outlined above, three different types of casein hydrolysate emulsions exist according to the particle size distribution of the emulsion droplets directly after homogenization. To investigate whether these particle size distributions are related to emulsion stability, regression analysis was performed with the size distribution as *x*-variables and the stability

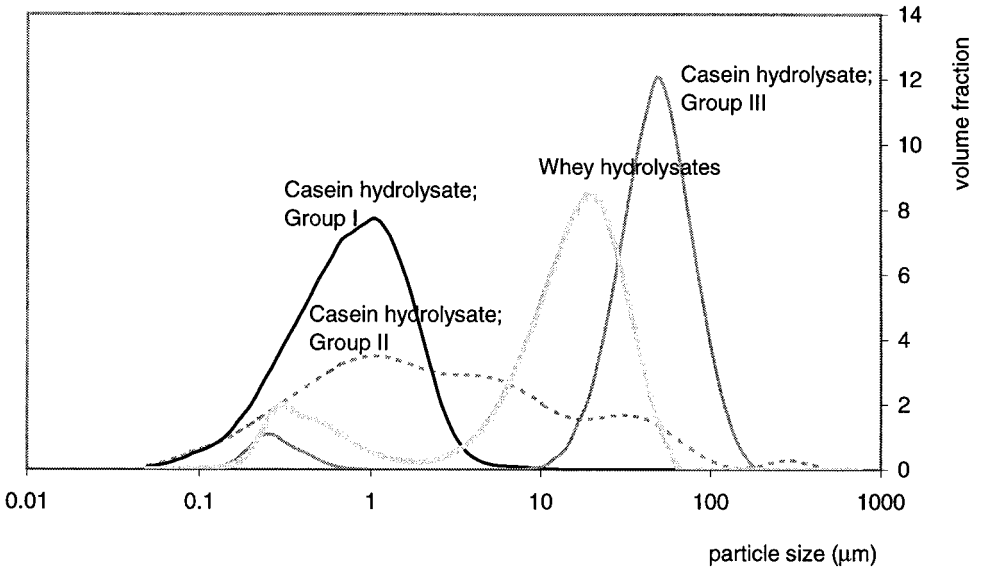

Figure 1. Particle size distributions of hydrolysate emulsions representing three types of casein emulsions and a typical whey hydrolysate emulsion. Distribution is expressed as vol % of oil included in droplets of each particle size. Samples used to represent groups were as follows: group I, CnCl102; group II, CnAlc14; group III, CnNwf10; whey, WcCl106.

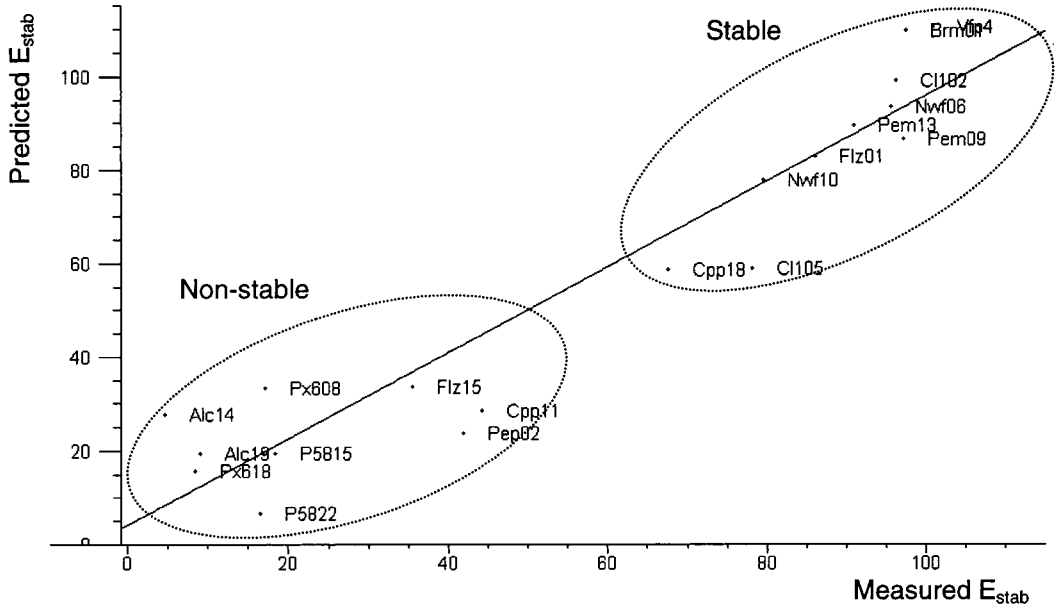

Figure 2. Correlation between predicted and measured emulsion stability (E_{stab}), expressed as remaining turbidity, of casein hydrolysate emulsions. Emulsion stability was predicted using particle size distribution curves of freshly made casein emulsion. Correlation coefficient between predicted and measured values is 0.94.

of emulsions measured by remaining turbidity as *y*-variable. The results revealed that the predicted emulsion stability based on particle size distribution correlates rather good with the measured emulsion stability (Figure 2); the correlation coefficient between the predicted and the measured values was 0.94.

The emulsions can be separated into a stable group and a nonstable group (Figure 2). When the particle size distributions of Figure 1 are correlated to the emulsion stability data, it can be concluded that stable emulsions are members of group I or group III and are characterized by a narrow particle size distribution. Emulsions from hydrolysates of group I have a typical low average particle size (d_{32}, 0.56−0.95 μm) whereas stable emulsions of group III have a rather large average particle size (d_{32}, 2−5 μm). Although the emulsions of group III are stable against coalescence, they are not stable against creaming of large droplets present immediately after homogenization. The hydrolysates forming nonstable emulsions have a very broad particle size distribution (hydrolysates from group II), or the emulsions are composed of very large droplets with d_{32} larger than 7 μm.

Peptides contribute to emulsion forming by lowering the interfacial tension, by facilitating droplet breakup, and by prevention of recoalescence (*13*). The broad particle size distribution of emulsions made with hydrolysates from group II indicates that these hydrolysates contain peptides that are very surface active, enabling the formation of small droplets, but contain insufficient peptides that are able to stabilize the emulsion droplets.

Concerning whey protein hydrolysates, stability of emulsions as calculated from turbidity data was also correlated to particle size distribution of the emulsion droplets. The correlation coefficient between measured

Table 4. Correlation Coefficients of Hydrolysate Characteristics for Emulsion-Forming Ability (d_{32}) and Emulsion Stability (E_{stab})

	casein		whey	
	d_{32}[a]	E_{stab}[b]	d_{32}	E_{stab}
DH	0.15	−0.63	0.11	−0.76
free amino acids	0.27	−0.16	−0.20	−0.26
MWD fractions				
>5 kDa	0.00	0.54	0.16	0.73
4−5 kDa	−0.32	0.52	0.01	0.81
3−4 kDa	−0.42	0.21	−0.26	0.70
2−3 kDa	−0.16	−0.14	−0.35	0.01
1−2 kDa	0.16	−0.58	0.10	−0.77
0.5−1 kDa	0.24	−0.63	−0.03	−0.78
<0.5 kDa	0.25	−0.70	−0.06	−0.76

[a] d_{32} is the volume−surface average particle size of the emulsion droplets. [b] E_{stab} is the stability of the emulsions (measured as remaining turbidity) over 24 h.

and predicted values was 0.84 (data not shown). Generally, whey hydrolysate emulsions showed a turbidity decrease of less than 40% and would be classified as stable emulsions in terms of casein hydrolysate emulsions. As already mentioned, instability due to creaming is more important in whey hydrolysate emulsions than instability due to coalescence.

Correlation of Emulsion Properties with Biochemical Properties of Protein Hydrolysates. As stated above, several authors suggested that emulsion properties of protein hydrolysates are related to their DH or to their peptide MWD. However, no consensus exists about these relations. The diverse results might arise from differences in peptide composition of the hydrolysates or from differences in analytical methods. As in the present study, all hydrolysates were made under similar conditions (pH-stat setup, standard protein source, and concentration), and as analytical methods were standardized, a general insight about the influence of DH, MWD, or enzyme specificity on emulsion properties can be obtained. To investigate these relations, statistical data analysis was used.

Correlation coefficients between hydrolysate characteristics and emulsion properties (Table 4) provide a first indication about important factors. Free amino acid content of hydrolysates was not related to emulsion properties as was shown by the low correlation coefficients. Degree of hydrolysis and molecular weight of peptides are factors often mentioned with regard to emulsion properties; therefore, these properties were studied in more detail.

Correlation between Emulsifying Properties and Degree of Hydrolysis. The calculation of correlation coefficients (Table 4) revealed that for both casein and whey hydrolysates no direct relation exists between DH and emulsion-forming ability as expressed by d_{32} values. In case of whey protein hydrolysates, with DH varying from 5.5 to 24%, all emulsions had similar droplet size distribution, confirming that the DH in that range does not correlate to emulsion-forming abilities.

According to the correlation coefficients for casein hydrolysate emulsions, no linear relation exists between d_{32} value and DH if all samples are analyzed together. As outlined before, three types of casein hydrolysate emulsions were distinguished based on the entire particle size distribution of emulsion droplets. The DH values of the hydrolysates belonging to the three groups are given in Table 5.

Hydrolysates forming narrow emulsion droplet size distributions with low average particle size (group I) all had DH ≤ 6%. Other low DH hydrolysates (made with other enzymes) formed emulsions composed of a broad range of droplet sizes (group II) or having a high amount of large particles (group III). Interestingly, if only samples of group III with d_{32} larger than 2.9 μm are considered, a linear relation between DH and d_{32} value exists (correlation coefficient = 0.94). For the other hydrolysates, resulting in emulsions with low d_{32} values (<1.1 μm), DH and d_{32} were not correlated.

Table 5. Degree of Hydrolysis and Enzyme Used To Prepare Hydrolysates of Three Types of Casein Hydrolysate Emulsions

	DH range	enzyme source[a]
group I	1−6	Brm, Cl1, Flz
group II	2−19	Alc, CPP, Px6, Pep
group III	4−22	Nwf, Pem, P58, Vfp, Flz, Pep

[a] For abbreviations see Materials and Methods.

From these results, it can be concluded that no overall correlation between DH and d_{32} value exists. The DH value alone does not include sufficient information about a hydrolysate to explain its emulsion behavior. A DH optimum for emulsion properties (*10*) or linear relation between DH and emulsion-forming ability (*9*) may therefore only be found if one protein/enzyme combination is considered.

Concerning the stability of emulsions, casein hydrolysates forming nonstable emulsions roughly had DH values >8% (data not shown). In general, low DH hydrolysates result in more stable emulsions than high DH hydrolysates, although some hydrolysates forming stable emulsions were hydrolyzed to DH values higher than 10%. The correlation coefficient also indicates that a general negative correlation exists between DH and emulsion stability. The decrease in emulsion stability with increasing DH is in agreement with literature (*8*, *16*, *31*).

Correlation between Emulsifying Properties and Apparent MWD of Hydrolysates. The correlation coefficients between molecular weight fractions and d_{32} (Table 4) reveal that, in case of whey protein hydrolysates, the volume−surface average particle size is not correlated to the MWD of the peptides in the hydrolysates. For casein hydrolysates, only the 3−4-kDa fraction shows some relation with d_{32}, signifying a decrease in emulsion droplet size with an increase of the proportion peptides of 3−4 kDa in the hydrolysate. However, the correlation coefficient is not significant.

For both casein and whey hydrolysate emulsions, a correlation between molecular weight fractions and emulsion stability measured as remaining turbidity was observed. All correlation coefficients, with the exception of the coefficients for 3−4- and 2−3-kDa fractions for casein and 2−3-kDa fraction for whey hydrolysates, were significant. A high proportion of high molecular weight peptides (larger than 3−4 kDa) is positively related to emulsion stability, while a high proportion of peptides smaller than 2 kDa (low amount of high molecular peptides) results in nonstable emulsions.

Regression analysis with molecular weight fractions as *x*-variables was performed to study these correlations in more detail. As the *x*-variables were interdependent, regression analysis was performed with multivariate principal component regression (PLS). The regression analysis with MWD and emulsion stability of casein hydrolysates resulted in a model with five principal components, which means that MWD data could be reduced to five "variables" describing the variance in

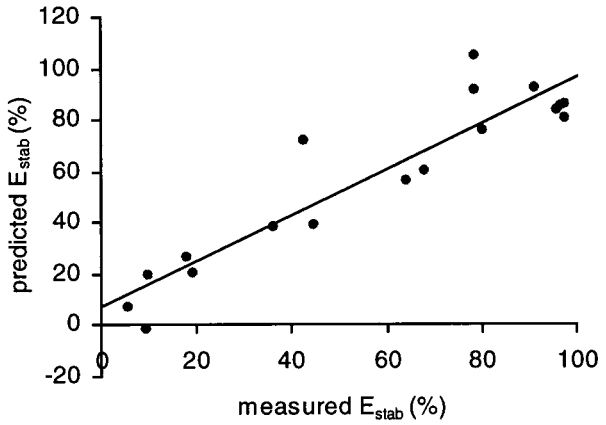

Figure 3. Correlation between predicted and measured emulsion stability (E_{stab}), expressed as remaining turbidity, of casein hydrolysate emulsions. Prediction was based on apparent molecular weight distribution of the hydrolysates. Correlation coefficient between predicted and measured values is 0.93.

emulsion stability. The correlation between measured and predicted values was 0.93 (Figure 3), signifying that emulsion stability is explained for a large extent by MWD of the peptides in a hydrolysate.

As was already seen with the correlation coefficients, the regression coefficients (data not shown) confirmed that generally a relative high proportion of peptides larger than 2 kDa are needed to form relative stable emulsions. The majority of the stable emulsions (turbidity decrease less than 40%) contained at least 65% peptides larger than 2 kDa (peptide chain length $\cong$17 amino acids). Exceptions on this rule were observed however. Two samples containing less than 65% peptides larger than 2 kDa were stable. The prediction of the emulsion stability based on the MWD of these samples was good, which might point to the importance of the presence of peptides with different molecular weights. Furthermore, it can be concluded that protease specificity is not important for emulsion stability since all hydrolysates containing high amounts of peptides >2 kDa show relatively high emulsion stability.

Concerning emulsion stability of whey protein hydrolysates, regression analysis with the molecular weight fractions resulted in a correlation coefficient of predicted versus measured emulsion stability of 0.90. As with casein hydrolysates, the peptides larger than 2 kDa were positively related to emulsion stability. As was mentioned before, creaming was the most important factor for instability of whey hydrolysate emulsions, which is not measured using the turbidity method. Although turbidity change only reflects the overall coalescence of emulsions, without providing information about the preceding mechanisms, it appeared to be a valuable method to correlate emulsion stability to MWD of hydrolysates.

Generally, high molecular weight peptides are mentioned to be beneficial for emulsion stability; the minimum size was estimated to be larger than 2 kDa (*11*, *37*), between 2.5 and 5 kDa (*38*) or larger than 5 kDa (*8*). The present study defined the relation between molecular weight of peptides and emulsion stability statistically, confirming that in general a high proportion of peptides larger than 2 kDa is needed. According to the presented results a hydrolysate containing mainly peptides smaller than 500 Da will not give a stable emulsion, which seems to contradict the results found by Singh and Dalgleish (*23*). However, only the average molecular weight of the hydrolysates was reported; hence, large peptides might also be present in the hydrolysates. Possibly the amount of large peptides is sufficient to stabilize the emulsions.

In the present study, a correlation was found between emulsion stability and MWD of hydrolysates. In a recent study, Rahali and co-workers (*26*) concluded that amphiphilicity is more important for emulsion properties than peptide length. However, emulsion forming and emulsion stability should be regarded as two separate processes. The adsorption of peptides on the interface reflects emulsion-forming ability of hydrolysates rather than emulsion stability. In the present study, it was shown that differences in d_{32} values were not related to MWD, which agrees with the study of Rahali and coworkers (*26*).

In conclusion, it was shown that the emulsion-forming behavior of hydrolysates is generally independent of MWD and DH. Emulsion instability was caused by creaming and coalescence. Creaming was observed in emulsions containing relatively large emulsion droplets, often already present directly after homogenization. Coalescence of emulsion droplets was correlated to the MWD of the hydrolysates stabilizing the emulsions. Hydrolysates with a high proportion of peptides with molecular weight larger than 2 kDa formed emulsions that are relatively stable toward coalescence.

ABBREVIATIONS USED

DH, degree of hydrolysis; MWD, molecular weight distribution; d_{32}, volume−surface average particle size; TNBS, trinitrobenzenesulfonic acid.

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