Biochemical and functional characterisation of casein and whey protein hydrolysates.

A study on the correlations between biochemical and functional properties using multivariate data analysis

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 4 juni 2002
des namiddags te vier uur in de Aula

ISBN: 90-5808-653-4

ABSTRACT

Van der Ven, C. (2002). Biochemical and functional characterisation of casein and whey protein hydrolysates. A study on the correlations between biochemical and functional properties using multivariate data analysis.
Ph.D. thesis Wageningen University, The Netherlands, 2002.

Keywords: Milk proteins, enzymatic hydrolysis, peptides, characterisation, chromatography, FTIR spectroscopy, emulsions, foams, ACE inhibition, multivariate data analysis, response surface methodology.

Whey protein concentrate and sodium caseinate were hydrolysed with commercially available enzyme preparations. The resulting hydrolysates were characterised using several analytical characterisation methods and by determination of several functional properties. Subsequently, correlations between the biochemical characteristics themselves and between biochemical and functional properties were studied using multivariate regression analysis.
Biochemical characteristics of hydrolysates were determined using unifactorial methods like the degree of hydrolysis, and by multifactorial methods, *i.e.* reversed phase (RPC) and size exclusion chromatography (SEC), and Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy appeared to discriminate most effectively between hydrolysates made from different protein sources and classes of proteolytic enzymes, followed by RPC and SEC.
Emulsion and foam properties of hydrolysates were similar or inferior to those of the parental proteins. Casein hydrolysates generally showed better emulsion- and foam-forming abilities than whey protein hydrolysates. Foam-forming ability of whey protein hydrolysates was correlated to the molecular weight distribution (MWD) of the peptides, showing that especially peptides with MW of 3-5 kDa contributed to foam-forming ability.
Concerning prevention of emulsion instability due to coalescence it was shown that peptides with a molecular weight larger than 2 kDa are needed. Foam-stabilising ability of casein hydrolysates also depended on the MWD of hydrolysates, but higher molecular weight peptides, *i.e.* larger than 7 kDa, were needed to obtain good foam stability.
The ability of the three multifactorial characterisation methods (SEC, RPC, FTIR spectroscopy) to predict functional properties was investigated. It appeared that SEC profiles were able to predict emulsion- and foam stability of all hydrolysates, as well as foam-forming ability, Angiotensin Converting Enzyme (ACE) inhibiting ability and bitterness of whey protein hydrolysates. RPC profiles were also able to predict these properties and additionally predicted solubility and bitterness of casein hydrolysates. FTIR spectra were best suited to predict a variety of hydrolysate properties, since apart from the before-mentioned properties, the spectra can also be used to predict emulsion-forming ability and to improve prediction of bitterness of hydrolysates.
Finally, the influence of hydrolysis process conditions on ACE-inhibiting activity of whey hydrolysates was investigated, showing that ACE-inhibiting activity could be optimised by using process optimisation techniques like experimental design and response surface optimisation.

Symbols and abbreviations

ACE	angiotensin converting enzyme
DH	degree of hydrolysis (%)
EAI	emulsifying activity index
FTIR	Fourier transform infrared spectroscopy
MW	molecular weight (kDa or Da)
MWD	molecular weight distribution
OPA	o-phthaldialdehyde
PCA	principal component analysis
PLS	partial least squares (multivariate regression)
RPC	reversed phase chromatography
SD	standard deviation
SEC	size exclusion chromatography
TNBS	trinitrobenzenesulphonic acid
WPI	whey protein isolate
WPC	whey protein concentrate

h	number of hydrolysed peptide bonds (meq/g)
h_{tot}	total number of peptide bonds in protein
p_ℓ	Laplace pressure (Pa)
γ	interfacial tension (N/m)
d_{32}	volume surface particle diameter (μm)
d_{43}	weight mean diameter (μm)
E_{stab}	emulsion stability (remaining turbidity after 24 hours (%)
F_0	foam volume after whipping (ml)
Vf_{60}	% foam volume remaining after 60 minutes relative to F_0
PreT	pre-treatment temperature
E/S	enzyme to substrate ratio (w/w)

Contents

CHAPTER 1

General Introduction

MILK PROTEINS

Proteins are important constituents of the human diet, since they are a principal source of nitrogen and essential amino acids. Milk proteins have a high nutritional value compared to other proteins because of their relative high content of essential amino acids and their good digestibility (Hambraeus, 1992). Moreover, milk proteins may contribute to structural properties of end products, such as emulsion, foam and gel properties. Milk proteins are used in many different food products, ranging from dairy products to beverages, dietary- and medical products (Mulvihill, 1992; Walstra et al., 1999). For some food applications proteins are hydrolysed, amongst others for hypoallergenic infant nutrition, for nutrition for patients with digestion disorders and for sports nutrition (Mahmoud, 1994; Swartz, 1995).

Caseins and whey proteins are the two main protein groups in milk (Table 1). Caseins, representing about 80% of the protein content in bovine milk, are isolated from milk by acid or by rennet precipitation. The acid, or isoelectric, precipitation is performed at pH 4.6, where the caseins precipitate and the whey proteins remain soluble. Acid precipitated caseins can be resolubilised by raising the pH through alkali addition, usually sodium hydroxide, yielding soluble caseinates. In rennet precipitation, one of the casein molecules, *i.e.* κ-casein, is selectively hydrolysed at the pH of milk (pH 6.7). As a result, the casein micelles in milk become susceptible towards precipitation with calcium. Rennet precipitation is used in cheese production. Casein consists of four primary proteins, *i.e.* α_{s1}-, α_{s2}-, β- and κ-casein, which all have genetic variants that differ in their molecular weight and isoionic pH. Caseins are flexible, heat stable, proteins. Typical for casein proteins is the clustering of amino acids in hydrophobic and hydrophilic domains, giving rise to the amphipathic character of the proteins (Mulvihill, 1992; Swaisgood, 1992).

Table 1: Milk proteins and their characteristics[a].

	% of total milk protein	MW range[b] (Da)	Estimated average MW (Da)	Isoionic pH
Casein	79-83			
α_{s1}-casein	30 -36	22066 - 23722	23600	4.91 - 5.35
α_{s2}-casein	8 -11	25148 - 25388	25200	5.19 - 5.39
β-casein	25 -28	23939 - 24089	24000	5.11 - 5.85
κ-casein	9 -10	19005 - 19037	19000/ 19550	5.37 - 6.07
γ-caseins[c]	2 - 4	11600 - 20500	20500	5.8 - 6.0
Whey protein	17-21			
β-lactoglobulin	9 -10	18205 - 18363	18300	5.14 - 5.49
α-lactalbumin	2 - 4	14147 - 14175	14000/ 14200	4.2 - 4.8
serum albumin	~1	66267 - 69000	66300	4.71 - 5.13
immunoglobulins	~2	153000 - 901000		5.5 - 8.3
proteose peptone	2 - 4	4100 - 40800		3.3 - 3.7
Miscellaneous	<2.5			

[a] Data obtained from (Marshall, 1982; Swaisgood, 1982; Eigel et al., 1984; Belitz & Grosch, 1987; Barth & Behnke, 1997; Walstra et al., 1999; Modler, 2000).

[b] MW ranges of caseins, β-lactoglobulin, α-lactalbumin and serum albumin result from differences between genetic variants of the proteins.

[c] Enzymatic hydrolysis products from β casein (C-terminus).

Whey proteins (or milk serum proteins) are defined as proteins in milk that remain soluble after acid (Walstra & Jennes, 1984) or after rennet casein precipitation (Barth & Behnke, 1997). The former whey protein source is known as acid whey, the latter is referred to as sweet- or rennet whey (Schmidt et al., 1984; Morr, 1989). Rennet whey contains the rennet proteolysis product of κ-casein, glycomacropeptide (GMP), which is obviously absent in acid whey (Barth & Behnke, 1997; Walstra et al., 1999).

Whey proteins are globular proteins that are soluble over a broad pH range (Mulvihill, 1992). The most important proteins in whey are β-lactoglobulin and α-lactalbumin, representing 70 to 80% of the protein content of whey (Walstra & Jennes, 1984; Morr, 1989; Smithers et al., 1996). Like caseins, different genetic variants of these proteins exist (Eigel et al., 1984). The remaining whey protein consists mainly of immunoglobulins, proteose peptones and bovine serum albumin (Schmidt et al., 1984; Smithers et al., 1996; Barth & Behnke, 1997). The proteose peptone fraction is mainly composed of degradation products of β-casein, which, at neutral pH, are present for a considerable part in the casein micelles. Therefore, rennet whey, which results from precipitation of casein at neutral pH, does not contain the entire proteose peptone fraction, whereas acid whey does, since at acidic pH the proteose peptones are no longer in the casein micelles (Walstra et al., 1999). β-Lactoglobulin is the most abundant whey protein and is rather sensitive to heat treatment. This protein contains one free thiol group, which may form new inter- and intramolecular bonds after modification of the protein by *e.g.* heat or proteolysis (de Wit & Klarenbeek, 1984; Hoffmann & Van Mil, 1997; Caessens et al., 1999a). α-Lactalbumin is a relatively small compact globular protein, which is stabilised by four intermolecular disulphide bonds (de Wit & Klarenbeek, 1984).

Pre-treatments of milk or whey, such as heat treatment or acidification, as well as the applied production methods, like precipitation, membrane fractionation, gel filtration and ion exchange chromatography, influence the composition of the final whey protein concentrates or isolates. Differences in protein compositions and protein conformations result in differences in the functional properties of the whey protein products (Schmidt et al., 1984; de Wit et al., 1986; Walstra et al., 1999).

ENZYMATIC PROTEIN HYDROLYSIS

Enzymatic protein hydrolysis is the degradation of proteins into peptides and/or amino acids by proteolytic enzymes. During protein hydrolysis amide bonds are cleaved, and, after addition of a water molecule, peptides and/or free amino acids are released (Figure 1). The newly formed peptides can be new substrates for the enzyme (Adler-Nissen, 1993).

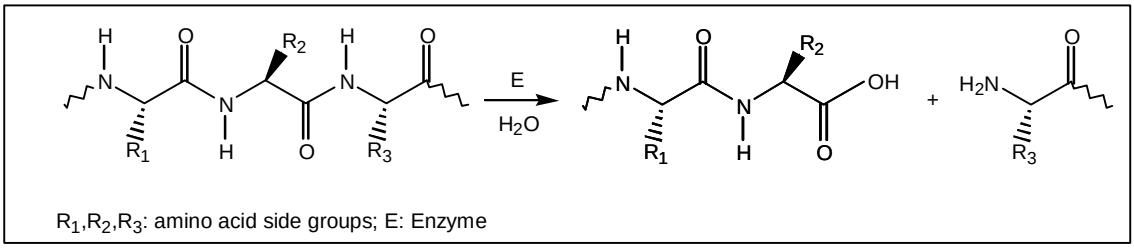

Figure 1: Hydrolysis process of proteins.

The hydrolysis process is proposed to occur as three consecutive reactions. First, a Michaelis complex of substrate (protein) and enzyme is formed, then the peptide bond is cleaved resulting in the liberation of one peptide, and finally, the remaining peptide is cleaved off the enzyme after a nucleophilic attack by a water molecule. These three steps are schematically depicted in Figure 2 (Adler-Nissen, 1993).

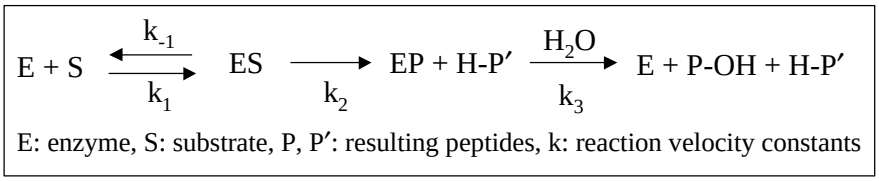

Figure 2: Catalytic mechanism of a protease (Adler-Nissen, 1993).

For protein hydrolysis, enzyme-substrate binding is essential. In case of globular proteins, most peptide bonds are located in the interior of the protein and are not accessible for the enzyme. For these globular proteins it was postulated by Linderstrøm-Lang that reversible denaturation of the protein is needed for protein breakdown, as after denaturation more peptide bonds are exposed. In solution the folded and unfolded (denaturated) states of proteins are in equilibrium. Only the unfolded molecules are susceptible to degradation by proteolytic enzymes, as schematically represented in Figure 3 (Adler-Nissen, 1986a).

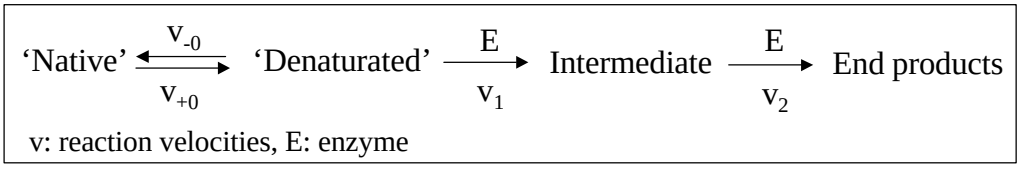

Figure 3: Linderstrøm-Lang theory (Adler-Nissen, 1986a).

If the rate of denaturation ($v_0=v_{+0}-v_{-0}$) is much smaller than v_1, the denaturation step is the rate limiting step for hydrolysis and each denatured protein molecule will be quickly hydrolysed to end products. The resulting hydrolysate will contain both intact proteins and end products, but will be deficient in intermediate size peptides. This type of reaction is designated as a 'one-by-one' reaction. If, otherwise protein denaturation is faster than hydrolysis ($v_1<v_0$), the protein molecules will be degraded to intermediates but are subsequently only slowly degraded to end products. This type of reaction is called a 'zipper' reaction, resulting in a hydrolysate containing mainly intermediate sized peptides. In most proteolytic reactions, both hydrolysis mechanisms are involved (Adler-Nissen, 1986a).

If the protein is irreversibly denaturated before hydrolysis, the number of accessible peptide bonds is largely increased and the degradation of the protein is expected to proceed according to a zipper type reaction. For these denaturated proteins other factors like decreased solubility might influence the initial reaction rate (Adler-Nissen, 1986a).

The result of proteolysis (the peptide composition of a hydrolysate) depends on three main factors: protein substrate, type of protease(s) used and hydrolysis conditions. These factors are discussed in the next paragraphs.

Protein substrate

Amino acid sequence and the three-dimensional structure of proteins affect their sensibility towards proteolytic attack and the type of peptides formed during hydrolysis. Caseins are rather flexible proteins and are, therefore, hydrolysed fairly easy (Swaisgood, 1992). Whey proteins on the contrary, are globular proteins that are difficult to access by proteolytic enzymes. Their digestibility can be improved by heat denaturation (Reddy et al., 1988; Guo et al., 1995). Another important difference between proteins is their primary amino acid sequence. In whey proteins hydrophobic and hydrophilic amino acids are randomly distributed over the peptide backbone, whereas caseins contain distinct hydrophobic and hydrophilic domains (Swaisgood, 1982). Hydrolysis of proteins with different distribution of hydrophobic and charged groups will result in peptides that differ in the distribution of hydrophobic/hydrophilic side groups. In a study of hydrolysates of β-lactoglobulin and β-casein it was shown that peptides obtained from β-lactoglobulin hydrolysis all showed rather similar distributions of charged and hydrophobic groups, lacking distinct hydrophobic or hydrophilic areas. On the contrary, the casein protein yielded peptides that differed in the distribution of charged and hydrophobic amino acids over the peptide sequence (Caessens, 1999).

Proteases

Many food-grade proteases are available for protein hydrolysis (Table 2). These proteases can be classified based on their origin, *i.e.* animal, plant or microbial origin, their mode of catalytic action, *i.e.* endo- or exo-activity, or on basis of their catalytic site. Endoproteases cleave amide bonds within the protein chain, contrary to exoproteases that remove terminal amino acids from proteins or peptides, either at the C-terminus (carboxypeptidases) or at the N-terminus (aminopeptidases). The nature of the catalytic site of proteases differs according to the active group that will form the enzyme/substrate intermediate. The active group can be either an amino acid, *i.e.* serine, cysteine or aspartic acid, or a metallo group, most often zinc (Adler-Nissen, 1993). The serine proteases are all endo-proteases, but metallo proteases are mostly exo-proteases (Whitaker, 1994). The pH specificity of proteases depends on the group in the catalytic site; the cysteine and metallo proteases are active at neutral pH, and the serine and aspartic acid proteases are active at alkaline and acidic pH, respectively (Adler-Nissen, 1993).

The amino acids present in the vicinity of the catalytic site are important for the substrate binding and determine which amino bonds can be potentially hydrolysed, thus determining the enzyme specificity (Adler-Nissen, 1993). The amino acid residues of the substrate at both sites of the cleaved amide bond are denoted P_1 and P'_1 for the carboxyl and amino sites, respectively (Figure 4) (Barrett et al., 1998).

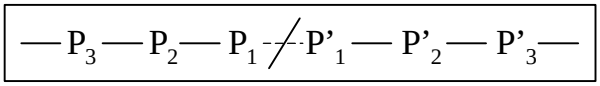

Figure 4: Denotation of amino acid residues at carboxylic (P_n) and amino (P'_n) site of cleaved amide bond (Barrett et al., 1998)

Table 2: Examples of proteases used for hydrolysis of food proteins[a]

Type of protease	Name	Source	pH-range	Preferential specificity[b]
Serine protease				
animal	Trypsin	Porcine, bovine	7-9	P_1: Lys, Arg
	Chymotrypsin		8-9	P_1: Phe,Tyr,Trp
	Elastase		6-8	P_1: Ala
bacterial	Subst. Carlsberg, Alcalase[c]	*Bacillus licheniformis*	6-10	Broad specificity, P_1 mainly hydrophobic a.a.
	Substilisin BPN, Substilisin Novo	*Bacillus amyloliquefaciens*	6-10	
Cysteine proteases				
plant	Papain	Papaya latex	5-8	Broad specificity, mainly P_2: hydrophobic a.a.
	Bromelain	Pineapple stem	5-8	
	Ficin	Ficus latex	5-8	
Aspartic protease				
animal	Pepsin	Porcine, bovine	1-4	P_1 and P'_1: Mainly hydrophobic a.a.
	Chymosin	Calf	4-6	P_1 and P'_1: Mainly hydrophobic a.a.
fungal	Chymosin-like	*Mucor pusillus, Mucor miehei, Endothia parasitica*	4-6	
	Aspergillo-peptidase A	*Aspergillus saitoi*	2-5	Preferably Glu, Asp, Leu
	Newlase	*Rhizopus sp.*	3-6	Like pepsin
Metallo protease				
animal	Carboxy-peptidase A	pancreas	7-8	Terminal a.a. at C-terminus of peptide, except Pro, Arg, Lys
bacterial	Neutral protease Neutrase[c]	*Bacillus amyloliquefaciens*	5-7	P'_1: Phe, Leu, Val
	Neutral protease, Thermolysin	*Bacillus thermoproteolyticus*	7-9	P'_1: Ile, Leu, Val, Phe
Technical preparations				
Mixture of papain, chymopapain, lysozyme	Crude papain	Papaya fruit	5-9	Broad specificity
Mixture of trypsin, chymotrypsin, elastase, carboxypeptidase	Pancreatin	Pancreas (bovine, porcine)	7-9	Very broad specificity
Mixture of serine-, aspartic-, metallo-protease	Veron P, Sumyzyme LP, Biozyme A	*Aspergillus oryzae*	4-8	Very broad specificity
Mixture of endo- and exo-proteases, active at alkaline and neutral pH	Pronase	*Streptomyces griseus*	7-9	Very broad specificity

[a] Data from (Adler-Nissen, 1993; Whitaker, 1994; Uhlig, 1998).

[b] P_n and P'_n represent preference for amino acid (a.a.) at carboxylic and amino site of cleaved bond, respectively (see Figure 4).

[c] Commercial preparations.

The difference in enzyme specificity for proteolytic enzymes belonging to the same group is exemplified by three endoproteases from animal origin, *i.e.* trypsin, chymotrypsin and elastase, which are all serine proteases (Table 2). These enzymes differ in their preferential specificity for the amino acid at the carboxylic side of the amide bond. Trypsin prefers either arginine or lysine residues, whereas chymotrypsin prefers aromatic amino acids (phenylalanine, tryptophan, tyrosine) and elastase prefers amino acids with small side-chains, such as alanine (Whitaker, 1994). Commercial enzyme preparations are often mixtures of different types of proteases, such as pancreatin that contains both endo- and exo-proteases, and crude papain which is a mixture of three proteases (Table 2).

Hydrolysis conditions

Once a protein/protease combination is selected, including a possible pre-treatment step of the protein, the reaction conditions of the hydrolysis process should be defined. The main variables determining the result of the reaction are temperature, pH, enzyme to substrate ratio and the reaction time. The first three factors determine the reaction rate and may influence the specificity of the enzyme mixture. The reaction time only determines the final extent of hydrolysis (Adler-Nissen, 1986a). The interactive effects between the hydrolysis parameters also influence the hydrolysate composition.

If the hydrolysis process is not controlled, the pH of the solution will change soon after the start of hydrolysis due to the formation of new amino- and carboxyl groups, which are able to release or accept protons, depending on the pH of hydrolysis. At low pH all amino groups are protonated and only part of the carboxyl groups are deprotonated, resulting in a net uptake of protons for each peptide bond cleaved, causing an increase of pH. At neutral and alkaline pH, hydrolysis results in a decrease of pH, since all carboxyl are deprotonated and only part of the amino groups are protonated. In order to prevent a pH change during hydrolysis, the reaction should be performed in a buffered system or in a pH-stat system in which the pH is maintained at the desired level by addition of acid or base (Adler-Nissen, 1986a).

CHARACTERISATION OF HYDROLYSATES

Due to protein hydrolysis, molecular properties of proteins change, like decreased molecular weight, increased charge, exposure of hydrophobic groups and disclosure of reactive amino acid side-chains (Nielsen, 1997; Caessens et al., 1999a). These molecular changes can be detected with several analytical methods, which reflect one or several molecular properties (Figure 5). As a result of the molecular changes, the functional properties of proteins are affected (Figure 5). Although the term functional property is often only applied to indicate techno-functional properties of hydrolysates, it should also comprise bio-functional properties, which can be subdivided in nutritional and physiological or biological functionality (Mahmoud, 1994) (Figure 5). Nutritional properties of hydrolysates reflect for example their increased digestibility and decreased allergenicity compared to the parental proteins. The physiological properties comprise potential bio-activities of hydrolysates, which originate from the liberation of bioactive peptides. Finally, the techno-functional properties represent technological functionality, such as solubility, foam and emulsion properties (Figure 5). Some

molecular characterisation methods and functional properties will be discussed in the next paragraphs.

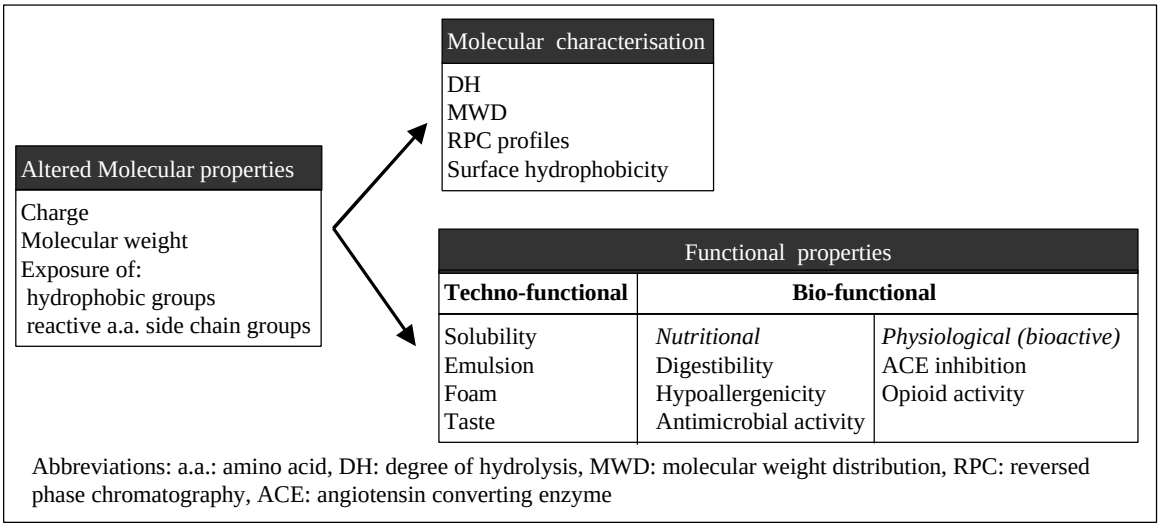

Figure 5: Changes in protein characteristics due to hydrolysis.

Molecular characterisation

The most commonly used parameter describing the result of a hydrolysis process is the degree of hydrolysis (DH), used as an indicator of the extent of hydrolysis. Another important parameter for protein hydrolysis is the molecular weight distribution of the peptides in hydrolysates. The molecular weight distribution is indicated by SDS-PAGE (Haque & Mozaffar, 1992; Perea et al., 1993; Guo et al., 1995; Galvao et al., 2001) or by size exclusion chromatography (Cordle et al., 1991; de Freitas et al., 1993; Boza et al., 1994; Boza et al., 1995; Sato et al., 1996; Lin et al., 1997; Madsen et al., 1997). These techniques are most often used to compare the hydrolytic action of various proteases, or to characterise hypoallergenic hydrolysates. Finally, hydrolysates are occasionally characterised by reversed phase chromatography (Haque & Mozaffar, 1992; Perea et al., 1993; Madsen et al., 1997), which yields detailed information about the complexity of hydrolysates, although direct comparison of hydrolysates using RPC profiles is difficult (Aubes-Dufau et al., 1995).

Degree of hydrolysis

Degree of hydrolysis represents the proportion of peptide bonds hydrolysed and is calculated according to equation 1, with h being the number of peptide bonds hydrolysed and h_{tot} being the total number of peptide bonds present in the parental protein. Both h and h_{tot} are expressed in meq/g (Adler-Nissen, 1986a).

$$DH = \frac{h}{h_{tot}} \times 100\% \qquad [1].$$

The h_{tot} of a protein is calculated from the protein amino acid composition (Adler-Nissen, 1986a). During hydrolysis, a new carboxyl and a new amino group is released for each cleaved amide bond (Figure 1). Therefore, the number of hydrolysed peptide bonds can be

deduced from the determination of the number of newly formed C- and/or N-terminal groups in hydrolysates.

As explained before, the amino and carboxyl groups are more or less (de)protonated after hydrolysis, depending on the pH of the solution. If hydrolysis is performed in a pH-stat set-up, the amount of added acid or base can be used to calculate the DH directly, since the addition of acid or base is related to the amount of liberated amino and carboxyl groups (Adler-Nissen, 1986a; Diermayr & Dehne, 1990). However, this method is only applicable for hydrolysis at neutral/alkaline (pH> 7) or acidic pH (pH< 3). At pH 5 to 6 there is no net release or uptake of protons, as the protonation and deprotonation of the acid/base groups are in equilibrium. Moreover, pK values, used to calculate the degree of dissociation of the acid/base groups, are not constant during hydrolysis since they depend on the peptide chain length and on the side chain of the terminal amino acid (Adler-Nissen, 1986a; Diermayr & Dehne, 1990; Camacho et al., 2001). Because of the problems occurring with determination of DH from pH-stat results, other methods to determine released amino groups are also needed.

The amount of released α-amino groups can be measured using reagents that react specifically with amino groups, yielding derivatives that can be detected spectrophotometrically. Reagents that are generally used are ninhydrin, *o*-phthaldialdehyde (OPA) and trinitrobenzenesulphonic acid (TNBS). Determination of the DH with these three reagents showed that results obtained with OPA and TNBS correlate well, whereas determination with ninhydrin resulted in much lower DH values (Panasiuk et al., 1998). Turgeon and coworkers (1991a) preferred the OPA method over the TNBS method as it was faster and more accurate. However, they measured the UV absorption of the OPA derivative immediately after the addition of the reagents. Panasiuk and coworkers (1998) later demonstrated that OPA absorption was stable only after 20 minutes. In the present study measurement of DH with the TNBS method was preferred since it was more reproducable and could be performed more efficiently than the OPA method.

Molecular weight distribution

From the degree of hydrolysis the average peptide chain length (PCL) of hydrolysates can be estimated according to equation 2, assuming that the entire hydrolysate is soluble (Adler-Nissen, 1986a).

$$PCL = \frac{100}{DH\,\%} \qquad [2].$$

The peptide chain length is related to the average molecular weight of peptides in hydrolysates. However, hydrolysates with similar PCL may have substantially different peptide molecular weight distributions. Enzyme specificity, for example, influences the peptide size distribution: a pure endoprotease will yield peptides with varying lengths, whereas an enzyme mixture that is mainly composed of exopeptidases will yield hydrolysates with mostly free amino acids combined with remaining large peptides. Moreover, proteases acting according to a one-by-one mechanism will yield hydrolysates with other peptide length distributions than proteases that hydrolyse proteins according to a zipper type reaction.

Molecular weight distribution of peptides in protein hydrolysates is generally determined using Size Exclusion Chromatography (SEC). With this chromatographic method molecules are separated principally on basis of the hydrodynamic volume, which depends on size and conformation. Unfortunately, separation is also influenced by so-called non-specific interactions between the proteinaceous components and the column material, like ion- and hydrophobic interactions (Visser et al., 1992; Silvestre et al., 1994; Fujinari & Manes, 1997; Smyth & Fitzgerald, 1997; Tossavainen et al., 1997). These interactions depend on the choice of eluents and column material (Barth, 1982; Smyth & Fitzgerald, 1997). The effect of non-specific interactions on the calculated apparent molecular weight distribution (MWD) can be reduced by inclusion of a large number of calibrating peptides that differ in their molecular properties (Visser et al., 1992).

Reversed phase chromatography

Another chromatographic method often used in the analysis of protein hydrolysates, is Reversed Phase Chromatography (RPC). Important for separation on RPC is the difference in hydrophobicity between amino acids. For small peptides (<15 residues), the hydrophobicity of the amino acid side chains in the peptides determine the elution time from a reversed phase column (Meek, 1980; Guo et al., 1986; Hearn et al., 1988). However, for larger peptides, the peptide length also influences retention time (Pearson et al., 1984; Mant et al., 1988; Chabanet & Yvon, 1992).

Generally, resolution on RP columns is better than on SEC. However, the results are more difficult to relate to molecular properties of the hydrolysates, since several characteristics, like peptide length, amino acid composition and presence of hydrophobic areas influence the retention time of peptides.

Functional characterisation

Bio-functional properties

Protein hydrolysates retain the amino acid composition of the parental protein, when the hydrolysate is not yet fractionated. However, digestibility and tolerance for proteins might be improved by protein hydrolysis, which can be regarded as improved nutritional properties (Figure 5). Moreover, the physiological functionality of proteins can be altered by the release of peptides with specific physiological properties (bioactive peptides).

<u>Nutritional properties</u>

An important nutritional reason to use protein hydrolysates is the increased digestibility of small peptides compared to whole proteins, which has been found to be especially beneficial for patients suffering from digestion disorders, such as cystic fibrosis, short bowel syndrome or pancreatitis (Rees et al., 1992; Frokjaer, 1994; Schmidl et al., 1994). A problem with consumption of protein products is the possible occurrence of allergic reactions, especially in infants towards *e.g.* milk proteins. Hydrolysis can be applied to destroy protein epitopes responsible for allergic reactions in sensitive individuals. For hypoallergenic products, proteins are usually extensively hydrolysed, thus resulting in products containing very low

molecular weight peptides (Pahud et al., 1985; Cordle et al., 1991; Boza et al., 1994; Martin Esteban et al., 1998; Halken et al., 2000).

Physiological properties

Beside nutritional aspects, proteins and peptides may exhibit specific physiological properties, like mineral binding or antimicrobial activity. Bioactive peptides may be present as such in the raw material, like growth factors in milk, or can be hidden in the primary sequence of proteins. The latter peptides can be released during protein hydrolysis. Several bioactive peptides have been found in milk protein hydrolysates, for example opioid, antihypertensive and antithrombotic peptides (Schlimme & Meisel, 1995; Korhonen et al., 1998; Xu, 1998; Clare & Swaisgood, 2000; Pihlanto Leppala, 2001). The antihypertensive effect of hydrolysates is related to inhibition of the Angiotensin Converting Enzyme (ACE). This enzyme is involved in blood pressure regulation as it converts the inactive peptide angiotensin I to the vasoconstrictive peptide angiotensin II. Moreover, the enzyme inactivates bradykinin, which is a vasodilatating peptide (Meisel & Schlimme, 1996; Clare & Swaisgood, 2000).

Inhibition of Angiotensin Converting Enzyme

The angiotensin converting enzyme is a zinc dependent metallopeptidase, which cleaves off C-terminal dipeptides from various oligopeptides. The enzyme has a broad substrate specificity and contains two substrate binding sites, with different affinity for substrates and/or inhibitors. Hydrolysis of substrates is often chloride dependent (Barrett et al., 1998). Peptides can act as ACE inhibitors according to different mechanisms. Firstly, peptides can bind to the active site of ACE without being hydrolysed by the enzyme. These peptides called true inhibitors. Secondly, inhibitor peptides can be substrates of ACE. These peptides are cleaved by ACE releasing new peptides that can either be less or more active inhibitors compared to the original peptides. The peptides yielding more effective inhibitors after cleavage by ACE are called prodrug type inhibitors, and peptides yielding less active inhibitors are called substrate type inhibitors. Examples of true inhibitor type peptides are LKP, IWH and IKP, whereas LKPNM and IWHHT are examples of prodrug type inhibitors (Fujita & Yoshikawa, 1999).

Numerous hydrolysates and peptides derived from food proteins have been reported to act as ACE inhibitors (reviewed by *e.g.* (Ariyoshi, 1993; Meisel & Schlimme, 1996; Yamamoto, 1997; Shah, 2000). Hydrolysates and fermentates of milk, casein and whey proteins exhibiting *in vitro* ACE inhibiting activity and/or *in vivo* antihypertensive effects are given in Table 3. In Table 4 amino acid sequences of peptides from whey and casein proteins reported to inhibit ACE are presented.

Hydrolysates prepared with different enzymes and from different protein sources result in *in vitro* ACE inhibition (Table 3), implying that ACE inhibition is induced by a variety of peptides and peptide combinations. The ACE-inhibiting activity depends on the protein source and the proteolytic enzymes used for hydrolysis. For example, in a study about hydrolysates of pancreatic enzymes, it was shown that hydrolysates of whey proteins prepared with elastase

Table 3: Examples of ACE inhibition by protein hydrolysates and fermentates of milk proteins.

Protein source	Proteolytic enzyme	*In vitro* ACE inhibition[a]	*In vivo* antihypertensive activity[b]	Ref[c]
WPC	Pepsin, pancreatic enzymes	36-89 %		1
β-Lactoglobulin	idem	63-88 %		
α-Lactalbumin	idem	57-87 %		
Whey protein	Actinase, trypsin	~55 %	SHR[d]: Improved effect of protein on SBP[d] after hydrolysis with Actinase, Trypsin, Proteinase K	2
	Chymotrypsin	76 %		
	Pepsin, papain	~85 %		
	Proteinase K, Thermolysin	~97 %		
α-Lactalbumin	Trypsin, pepsin + pancreatic enzymes	0.11-1.49 mg/ml		3
β-Lactoglobulin	Pepsin, trypsin, pepsin + pancreatic enzymes	0.24-1.73 mg/ml		3
Casein	Alcalase, trypsin	1.7, 0.55 mg/ml		4
Casein (Extracts of Cheese)	Total	12-73 %		5
	Fraction< 1 kDa	8 - 68 %		
Casein	Trypsin	0.17 mg /ml	SHR: SBP ↓	6
Milk	Yeast protease	0.42 mg /ml		7
Milk	*L. helveticus*[e] + *Saccharomyces cerevisiae*	—	SHR: SBP ↓	8
Milk	*L. helveticus* fermentation	—	SHR: SBP ↓	9

[a] ACE inhibition given as IC_{50} value (mg/ml) or as % ACE inhibition.
[b] Based on a single dose administration of hydrolysate.
[c] References: 1 (Mullally et al., 1997a), 2 (Abubakar et al., 1998), 3 (Pihlanto Leppala et al., 2000), 4 (Hyun & Shin, 2000), 5 (Meisel et al., 1997), 6 (Karaki et al., 1990), 7 (Roy et al., 2000), 8 (Nakamura et al., 1995b), 9 (Yamamoto et al., 1999).
[d] Abbreviations: SHR, spontaneously hypertensive rats; SBP, systolic blood pressure.
[e] *Lactobacillus helveticus*.

were less effective ACE inhibitors than hydrolysates from other pancreatic enzymes. Hydrolysis with trypsin generally results in hydrolysates with good ACE-inhibiting activity (Mullally et al., 1997a), but does not always yield hydrolysates with the best ACE inhibition (Abubakar et al., 1998; Hyun & Shin, 2000). Apart from enzyme specificity, hydrolysis conditions such as hydrolysis time and enzyme to substrate ratio also determine the final ACE-inhibiting activity (Matsui et al., 1993). For some proteases high ACE inhibition is reached after short hydrolysis times, yet with other enzymes longer hydrolysis time is needed (Lee et al., 1999). ACE inhibition by hydrolysates seems to be mainly caused by low molecular weight peptides, as permeate fractions obtained after filtration over 1 kDa membranes yield relatively high ACE inhibition (Meisel et al., 1997; Pihlanto Leppala et al., 2000).

ACE inhibiting activity *in vitro* does not necessarily result in a high antihypertensive activity *in vivo*. Nevertheless, only a few *in vivo* studies with milk protein hydrolysates were reported (*e.g.* (Karaki et al., 1990; Nakamura et al., 1995b; Abubakar et al., 1998; Yamamoto et al., 1999). In these studies a single dose of the potentially active hydrolysate is given to spontaneously hypertensive rats (SHR) and systolic blood pressure (SBP) is measured over a 24 hours period. The difference that may exist between *in vitro* ACE inhibition and *in vivo* blood pressure decrease is illustrated by a study on various whey protein hydrolysates

(Abubakar et al., 1998). This study showed that hydrolysates from typsin and Actinase (a fungal proteinase from *Actinomyces* spp.) having a relatively low *in vitro* ACE inhibiting activity, result in a relative large blood pressure decrease in SHR (Table 3).

ACE inhibiting peptides (showing *in vitro* inhibiting activity) derived form milk proteins have been purified or synthesised by several researchers (Table 4). Casein peptides seem to be slightly more effective than whey protein peptides. Some of the casein peptides were tested *in vivo* (in SHR) and indeed showed a decrease in blood pressure (Table 4). The reported ACE inhibiting peptides largely differ in their amino acid sequences. Small differences in amino acid sequence can largely affect the ACE-inhibiting activity (Table 4). Reports concerning structure-activity studies on ACE-inhibiting ability of peptides showed that the C-terminal tripeptides strongly influences ACE inhibition. Presence of proline, lysine and arginine (Meisel & Schlimme, 1996) as well as that of tryptophan, tyrosine, phenylalanine and proline (Cheung et al., 1980) at the C-terminus were reported to promote ACE inhibition. However,

Table 4: ACE inhibition by peptides derived from whey and casein proteins.

Protein source	peptide	ACE inh. (IC_{50} (μM)[a]	Ref[b]	Protein source	peptide	ACE inh. (IC_{50} (μM) [a]	Ref[b]
α-La[c]	YG	1522	1	Casein	YP[d]	720	5
	YGL	409	2		KVLPVPQ[d]	1000	6
	YGLF	733	1	β-Casein	FP[d]	315	3
	WLAHK	77	2		VYP[d]	288	3
	VGINYWLAHK	327	2		VYPFPG[d]	221	3
β-Lg[c]	YL	122	1		YPFPGPI	500	7
	LF	349	1		VPP[d]	9	8
	YLLF	172	1		IPP	5	8
	IR	696	1		TPVVVPPFLQP	749	3
	RL	2439	1		LQSW	500	6
	HIRL	1153	1		DKIHPF	257	9
	IPA[d]	141	3		AVPYPQR[d,e]	15	10
	VFK	1029	2		LNVPGEIVE	300	9
	LAMA	1062	2		NIPPLTQTPV	173	9
	ALPMH	521	2		YQQPVLGPVR	300	7
	ALPMHIR	43	4	α_{s1}-Casein	YKVPQL	22	6
	CMENSA	788	2		AYFYPE	106	11
	VLDTDYK	946	2		FFVAP	6	10
	LDAQASPLR	635	2		FPEVFGK	140	7
					FFVAPFPEVFGK[d,e]	77	12
				α_{s2}-Casein	TKVIP	400	6
					TTMPLW[d,e]	16	13
					AMKPW	580	6
					MKPWIQPK	300	6
					AMKPWIQPK	600	6
				κ-Casein	IPP[d]	5	8

[a] Concentration of peptide needed to reduce ACE activity by 50%.

[b] References: 1 (Mullally et al., 1996), 2 (Pihlanto Leppala et al., 2000), 3 (Abubakar et al., 1998), 4 (Mullally et al., 1997b), 5 (Yamamoto et al., 1999), 6 (Maeno et al., 1996), 7 (Meisel, 1997), 8 (Nakamura et al., 1995a), 9 (Gobbetti et al., 2000), 10 (Maruyama et al., 1985), 11 (Yamamoto et al., 1994), 12 (Maruyama & Suzuki, 1982), 13 (Maruyama et al., 1987).

[c] α-La: α-lactalbumin, β-Lg: β-lactoglobulin.

[d] Peptides also showed blood pressure decrease in spontaneously hypertensive rats.

[e] Antihypertensive effect measured by Karaki and coworkers (1990).

based on the large diversity of reported ACE inhibitors, ACE-inhibiting activity seems to be rather a-specific. The most general factor in ACE inhibiting ability seems to be the low MW of the ACE inhibiting peptides.
It should be noted that the assay conditions used to study the *in vitro* ACE inhibiting ability of peptides are not standardised. Amongst others, the amount of ACE used in the assays as well as the type of ACE substrate are important for the calculated IC_{50} value (the peptide concentration needed to reduce the ACE activity by 50%). However, often the ACE concentration is not even mentioned. Differences in the assay conditions may be responsible for differences between IC_{50} values reported for the same peptide. For example, the peptide LKPNM was reported to have an IC_{50} value of 17 μM (Yamamoto, 1997) as well as 2.4 μM (Fujita & Yoshikawa, 1999).

Techno-functional properties
Molecular changes occurring during protein hydrolysis may result in modified techno-functional behaviour of the hydrolysates compared to the intact protein such as altered solubility, viscosity, sensory properties, emulsion and foam properties (Panyam & Kilara, 1996; Nielsen, 1997; Caessens et al., 1999a). As emulsion and foam properties were studied in more detail in the present study, these properties will be discussed to a larger extent than the other mentioned properties.

Solubility
Generally, the solubility at the isoelectric point (pI) of proteins increases with hydrolysis, which is mainly the result of reduction in molecular weight and the increase in the number of polar groups (Chobert et al., 1988b; Nielsen, 1997; Slattery & Fitzgerald, 1998). The effect of hydrolysis on solubility at other pH values depends on the protein studied. Caseinates for example, are very soluble at pH values above and below the pI (pH 4-5). Consequently, at these pH values the solubility of hydrolysates is similar to or slightly lower than that of intact caseinates (Chobert et al., 1988a; Chobert et al., 1988b; Svenning et al., 1993; Slattery & Fitzgerald, 1998). Whey protein, which is, except at the pI, slightly less soluble than casein, shows increased solubility with hydrolysis over the entire pH range (Chobert et al., 1988a; Perea et al., 1993; Lieske & Konrad, 1996).

Bitterness
An important negative side effect of protein hydrolysis, is the liberation of bitter tasting peptides from the protein (Adler-Nissen, 1987). For bitterness of pure peptides it has been shown that the presence and position of hydrophobic amino acids are related to bitterness (Ney, 1979; Otagiri et al., 1985; Ishibashi et al., 1988; Tanimoto et al., 1992; Lovsin Kukman et al., 1995). Bitterness of hydrolysates is more complex and is considered to be mainly caused by small hydrophobic peptides in the hydrolysate (Adler-Nissen, 1986b; Lovsin Kukman et al., 1996). Development of bitterness in hydrolysates depends on the protein source and enzyme specificity (Petrischek et al., 1972; Vegarud & Langsrud, 1989; Tanimoto et al., 1992). Casein hydrolysates are especially known for their peptide bitterness (Lemieux

& Simard, 1992). Several analytical methods have been developed to predict bitterness of hydrolysates. Isolation of hydrophobic peptides by butanol extraction and subsequent determination of the hydrophobicity and average molecular weight of these peptides has been proposed by Adler-Nissen, resulting in rather good prediction of bitterness (Adler-Nissen, 1986b). Chemical parameters (Vangtal & Hammond, 1986; Frister et al., 2000) and infrared spectroscopy (Sorensen & Jepsen, 1998) have also been used to predict bitterness, especially for cheese samples.

Emulsions and foams

Emulsions and foams are both mixtures consisting of at least two immiscible phases. Foams consist of air bubbles dispersed in a continuous phase, for example water. Emulsions are oil-water mixtures, in which either the oil is dispersed as droplets in a continuous water phase (oil in water emulsions) or in which water is dispersed in the continuous oil phase (water in oil emulsions) (Damodaran, 1997). Formation of these dispersed systems requires energy input and the presence of surfactants, since the two phases do not mix spontaneously. The surfactants should be soluble in the continuous phase, which implies that if (water soluble) proteins or peptides are used as surfactants, the oil phase will be dispersed in the continuous water phase (Walstra & de Roos, 1993).

Surfactants are also involved in the stabilisation of the two phase-systems once they are formed. The physical role of surfactants in the formation and stabilisation processes is different. Therefore, formation and stabilisation of foams and emulsions should be regarded separately (Wilde & Clark, 1996; Damodaran, 1997; Walstra & Smulders, 1997). However, in case of foams a clear separation between formation and stabilisation is not possible, since instability phenomena will already occur during foam formation (Walstra, 1996).

Emulsion and foam formation

During emulsion or foam formation, three processes occur simultaneously: i) droplets are deformed and possibly broken-up, ii) surfactants move to and adsorb onto the newly formed interfaces, and iii) droplets collide, possibly resulting in coalescence. The break-up of droplets into smaller ones is counteracted by the Laplace pressure (p_ℓ), which is a function of the radius of the droplet (R) and the interfacial tension (γ) (equation 3).

$$p_\ell = \frac{2g}{R} \qquad (Pa) \qquad [3].$$

As can be seen from equation 3, the Laplace pressure increases with decreased droplet radius, thus hindering droplet break-up of small droplets. Addition of a surfactant lowers the interfacial tension, thus facilitating droplet break-up (Walstra, 1993).

Apart from influence on droplet break-up, the surfactant is also important for the prevention of coalescence. This occurs if the film between two droplets is ruptured and two droplets join to form a single larger droplet. Coalescence of droplets during emulsion and foam formation is mainly opposed by the formation of interfacial tension gradients (γ-gradient) which is brought about by two closely related mechanisms occurring simultaneously. First, if droplets or bubbles are pushed together during the formation process, liquid in the thin layer between

the droplets or bubbles will flow out. The liquid flow will sweep down surfactant molecules along the interface, resulting in a higher concentration of surfactants downstream. The induced γ-gradient subsequently counteracts the liquid flow and will slow down the mutual approach of droplets or bubbles. Although the effect of a γ-gradient may only act during a short time, it might be long enough to drive droplets/bubbles apart (Walstra & Smulders, 1998; Van Kalsbeek & Prins, 1999). In addition to the liquid flow causing a γ-gradient, the Gibbs-Marangoni effect may occur. This effect describes the formation of an interfacial tension gradient due to the fact that the amount of surfactant in the thin film between two approaching droplets is limited. During approach of the droplets/bubbles surfactants will continue to adsorb on the interface. As the amount of surfactant in the thin film between two approaching droplets is lower than in the bulk phase, adsorption of additional surfactants onto the interfaces of the film will be less than onto other parts of the droplet interface. This will cause the formation of an interfacial tension gradient. This γ-gradient subsequently causes a liquid flow into the film. The Gibbs-Marangoni effect only occurs when emulsion droplets are not yet saturated with surfactants (Walstra & Smulders, 1997; Walstra & Smulders, 1998; Van Kalsbeek & Prins, 1999).

An important difference between emulsion and foam formation is the time scale of the process. The homogenisation process of emulsification, with a characteristic time scale of 10^{-6} seconds, is more dynamic than the making of foams (time scale ~10^{-3} seconds). Consequently, the surfactants in emulsions have less time to adsorb onto and eventually rearrange on the surface (Dickinson, 1992; Walstra, 1996). Other important differences between foams and emulsions are particle size (about 1 mm for foams and 1 μm for emulsions) and difference in solubility of the dispersed phase in the continuous phase (solubility of air in water is higher than solubility of oil in water) (Walstra, 1996).

Emulsion and foam stability

Once an emulsion or foam is formed, it is subject to several instability processes. For both foams and emulsions three important causes of instability can be distinguished. These three causes result in three different instability phenomena, of which two occur in both systems (Table 5). The first listed cause of instability is the density difference between the dispersed and continuous phases. This may result in separation of the dispersion in an upper layer containing a high concentration of dispersed particles and a lower layer with the continuous phase.

Table 5: Instability factors of dispersions and the effect on emulsions and foams.

Cause of instability	**Instability phenomenon**	
	Emulsion	**Foam**
Density difference between dispersed and continuous phase	Creaming	Creaming, drainage
Attraction between surfactants on interfaces of different droplets	Aggregation	
Film rupture	Coalescence	Coalescence
Laplace pressure		Ostwald ripening

This process is referred to as creaming at low disperse phase volume fraction (φ) and drainage at high φ (Hill, 1996). Creaming velocity, indicated by Stokes' law (equation 4), depends on the particle diameter (d), on the density difference between dispersed and continuous phases (Δρ), on the acceleration due to gravity (g) and on the continuous phase viscosity (η_0) (Hill, 1996).

$$velocity = \frac{g\,\Delta r\,d^2}{18h_0} \qquad (m/s) \qquad [4].$$

Emulsions are stable towards creaming if the emulsion droplets are sufficiently small, circa 1 μm, since the creaming is then opposed by the Brownian movement. Creaming of foams occurs rapidly after beating stops, due to the large density difference between air and the continuous phase and due to the relatively large bubble diameter. After creaming, liquid starts to drain out of the foam layer (Walstra, 1996).

The second mechanism of instability, occurring only in emulsions, is caused by attraction forces between surfactants on the interfaces of different droplets, the individual droplets remaining intact. Attraction is promoted by van der Waals and hydrophobic interactions as well as by electrostatic attraction, but it is opposed by electrostatic and steric repulsion. The result is aggregation of emulsion droplets, which may lead to creaming (Wilde & Clark, 1996; Damodaran, 1997).

The third cause of instability listed is film rupture, which occurs in both emulsions and foams, leading to coalescence of droplets or bubbles. Coalescence is irreversible, as the film is broken and larger new droplets are formed. Creaming and aggregation might promote coalescence, as the droplets are close to each other for a prolonged time (Damodaran, 1997; Smulders et al., 1999).

Although creaming/drainage and coalescence do occur in foams, the main cause of instability in foams is Ostwald ripening (disproportionation). This instability is caused by the diffusion of air from small to large air bubbles, resulting from the higher Laplace pressure in small bubbles compared to that in large ones (Walstra, 1996; Damodaran, 1997). Ostwald ripening does not occur in emulsions, since the solubility of oil in water is much lower than the solubility of air in water (Walstra, 1996).

Molecular properties affecting emulsions and foams

Emulsions and foams can be created and stabilised in the presence of high molecular weight surfactants like proteins and/or low molecular weight surfactants like small peptides. The molecular properties required for the formation of dispersed systems differ from properties required for stabilisation of these systems. Moreover, the molecular properties required for stable emulsions may differ from those required for stable foams.

For formation of dispersed systems, surfactants should be able to decrease the interfacial tension and prevent immediate coalescence. For the first function surfactants should be able to migrate and adsorb quickly to the interface. Small molecules will diffuse and adsorb quickly, whereas in the case of proteins their molecular flexibility influences the rate of adsorption (Wilde & Clark, 1996; Damodaran, 1997; Walstra & Smulders, 1997). For prevention of

immediate coalescence the ability to create a γ-gradient is essential, which can be attained by most sorts of surfactants (Walstra, 1996).
For emulsion stability purposes, electrostatic and steric hindrance are often mentioned as important factors that prevent aggregation or coalescence of droplets (Wilde & Clark, 1996; Damodaran, 1997). In case of foams, where disproportionation and drainage are the main causes of instability, the major role of surfactants is to remain adsorbed to the interface when maintaining a γ-gradient. In case of disproportionation, the air bubble shrinks, decreasing the interfacial area, resulting in an increase of surfactant concentration. If surfactants remain adsorbed, γ decreases, resulting in lower Laplace pressure, thereby diminishing the driving force of disproportionation (Walstra, 1996). Drainage of liquid from films is opposed by the formation and maintenance of γ-gradients (Prins, 1987). This implies that the surfactants should remain adsorbed at interfacial areas of high surfactant concentration (low interfacial tension). Proteins that are attached to interfaces at several points desorb slowly from interfaces, whereas low molecular weight surfactants desorb easily (Walstra & de Roos, 1993). Interactions between neighbouring proteins or peptides will slow down desorption, thereby promoting foam stability (Prins, 1999).

Emulsion and foam properties of hydrolysates
Emulsion and foam properties of protein and protein hydrolysates depend on the pH of the system. Reports concerning the altered emulsion and foam properties of casein or whey protein hydrolysates compared to the parental protein, measured at neutral and alkaline pH, are summarised in Table 4. Data on molecular properties, like DH and molecular weight range of the studied hydrolysate fractions, are also included.
For both whey protein and casein, hydrolysis has been reported to improve as well as to reduce emulsion-forming properties (Chobert et al., 1988a; Chobert et al., 1988b; Haque & Mozaffar, 1992; Agboola & Dalgleish, 1996; Slattery & Fitzgerald, 1998). The effect of hydrolysis sometimes seems to depend on the enzyme used for hydrolysis. Chobert and coworkers, for example, reported improved emulsion formation for tryptic hydrolysates of casein (Chobert et al., 1988a), which is in agreement with results found by Haque and Mozaffar (1992). Otherwise, casein hydrolysates of *Staphylococcus Aureus* V8 protease showed decreased emulsion-forming activity (Chobert et al., 1988b). Hydrolysis with the same enzyme does not always result in the same effect on emulsion-forming behaviour, as is illustrated by the tryptic hydrolysis of β-lactoglobulin. Both increased (Vojdani & Whitaker, 1994; Caessens et al., 1999d) as well as decreased (Agboola & Dalgleish, 1996) emulsion-forming ability have been reported. These differences might result from differences in the DH of the hydrolysates or from differences in the methods used to prepare the emulsions and to quantify the emulsion-forming properties. For example, the emulsion activity index (EAI) measures the turbidity of an emulsions with a certain oil fraction, whereas the emulsifying capacity (EC) determines the amount of oil that can be dispersed by a certain amount of protein or hydrolysate. Although both methods are used to quantify emulsion-forming ability of hydrolysates, they actually do not measure the same properties. A tryptic casein

hydrolysate indeed showed increased emulsion-forming properties compared to the parental casein if emulsion-forming ability was measured as EAI, but showed decreased emulsion-forming properties if characterised by the emulsifying capacity (Chobert et al., 1988a).
Hydrolysis of whey protein and casein generally resulted in increased foam-forming ability of the hydrolysates compared to the parental proteins (Britten et al., 1994; Ludwig et al., 1995; Lieske & Konrad, 1996; Caessens et al., 1999d). In two studies whey protein hydrolysates were fractionated with ultrafiltration over a 10 kDa membrane (Althouse et al., 1995; Mutilangi et al., 1996). For almost all hydrolysates it appeared that the permeate fraction, with peptides having a MW <10 kDa, formed a higher foam overrun or faster a predefined foam level, than the original protein. The foam-forming behaviour of the retentate fraction decreased for some hydrolysates, although for trypsin, chymotrypsin and Alcalase the results differed between the two studies (Table 4).
A decrease in emulsion stability upon hydrolysis of casein using various enzymes has been observed by several authors (Chobert et al., 1988a; Chobert et al., 1988b; Haque & Mozaffar, 1992), although, Ludwig and coworkers (1995) reported improved emulsion stability. For β-lactoglobulin most reported hydrolysates showed improved emulsion stability (Vojdani & Whitaker, 1994; Caessens et al., 1999d). Hydrolysates of whey protein isolate (WPI) prepared with pancreatic enzymes (trypsin or chymotrypsin) or bacterial enzymes (Alcalase or Neutrase) were fractionated over a 10 kDa membrane, to study the differences in functional properties between total hydrolysates and high and low molecular weight peptide fractions (Mutilangi et al., 1996). Total tryptic and chymotryptic hydrolysates and their retentate fractions (MW>10 kDa) showed increased emulsion stability relative to WPI. The same applied for Alcalase or Neutrase hydrolysates with DH levels higher than 6%. With all tested hydrolysates, the fractions with peptides <10 kDa showed decreased emulsion stability compared to WPI. These results suggest that high molecular weight peptides have an essential role in emulsion stabilisation (Mutilangi et al., 1996). The positive effect of high molecular weight peptides on emulsion stability also appeared from a study concerning different fractions of a plasmin hydrolysate of β-lactoglobulin. A fraction containing peptides of 7 to 14 kDa showed improved emulsion stability relative to the parental protein, but an emulsion prepared with a fraction containing peptides <2 kDa was not able to stabilise the emulsion (Caessens et al., 1999a).
The effect of hydrolysis on foam stability seems to depend on enzyme specificity and degree of hydrolysis (Althouse et al., 1995; Ludwig et al., 1995; Mutilangi et al., 1996; Slattery & Fitzgerald, 1998). In case of tryptic whey protein hydrolysates, it was shown that the fraction with peptides having MW <10 kDa had better foam-stabilising properties than the peptides with MW >10 kDa (Althouse et al., 1995; Mutilangi et al., 1996). Other hydrolysates fractionated over a 10 kDa membrane, showed for both the high and low molecular weight fractions either increased or decreased foam stability relative to WPI.

Table 4: Emulsion and foam properties of casein and whey hydrolysates at neutral pH, compared to the functional properties of the parental proteins.

Measure	Protein	Enzyme	DH (%)	MW fraction	Ref [a]	Protein	Enzyme	DH	MW fraction	Ref
	DECREASED EMULSION FORMING ABILITY					*INCREASED/ SIMILAR EMULSION FORMING ABILITY*				
EAI	Na caseinate	Protamex	0.5, 1, 9, 15		1	Casein	Trypsin	4, 8, 10		9
	Casein	*Staph.aur.* V8 [b]	2, 7		2	Casein	Papain	?, low		4
	Casein	Corolase PS, Neutrase, Maxatase	1-4		3	Acid casein	Trypsin, Chymotrypsin, Rhozyme	2-5		13
	Casein	Alcalase, Chymotrypsin	?[c], low		4	β-Lactoglobulin	Endoprotease Arg-C	2		6
	Acid casein	Pancreatin	> 20		5	β-Lactoglobulin	Endoprotease Lys-C, Endoprotease Glu-C	5		6
	β-Lactoglobulin	Endoprotease Glu-C	7		6	β-Lactoglobulin	Trypsin	3, 10		6
	WPI	Alcalase, Neutrase	3-8	TH[d], <, >10kDa	7	WPC (79% protein)	Papain	3	TH, >1 kDa	14
		Trypsin, Chymotrypsin		<10 kDa	7	WPI	Trypsin, Chymotrypsin	3-8	TH, >10 kDa	7
	WPC (sweet whey)	Chymotrypsin,Trypsin	div. time		8	WPC 80	Trypsin	2.5, 4, 5		9
EC	Casein	Trypsin	4, 8, 10		9	WPC (79% protein)	Papain	3	TH, >1kDa	14
	WPC (gel filtration)	Prolase, pronase, pepsin	div. time		10	WPC 80	Trypsin	2.5, 4, 5		9
	WPC (35% protein) EC at high [I][f]	Trypsin, Chymotrypsin	E/S[e]=1/200 t=45 min	TH, >1kDa	18	WPC (35% protein) EC at low [I]	Trypsin, Chymotrypsin	E/S=1/200 t=45 min	TH, >1kDa	18
Particle size distribution	Na caseinate β-Lactoglobulin	Trypsin	?		11	β-Lactoglobulin	Trypsin, Plasmin, *Staph. aur*. V8	1, 2, 4		15
	β-Lactoglobulin	Plasmin	4	< 2 kDa[g]	12	β-Lactoglobulin	Plasmin	4	7-14 kDa[c]	12
	DECREASED FOAM FORMING ABILITY					*INCREASED/ SIMILAR FOAM FORMING ABILITY*				
Expansion						Na caseinate	Protamex	0.5-1		1
Foam capacity	Casein	Chymotrypsin	?, low		4	Casein	Alcalase, Papain	?, low		4
Overrun	WPI (95% protein)	Pepsin, Acid fungal protease	~3	>10 kDa	16	WPI (95% protein)	Alcalase, Chymotrypsin, Trypsin	~3	<, >10 kDa	16
	β-Lactoglobulin	Endoprotease Arg-C	2		6	WPI (95% protein)	Pepsin, Acid fungal prot.	~3	< 10 kDa	16
						β-Lactoglobulin	Endoprotease Glu-C	5, 7		6
						β-Lactoglobulin	Trypsin	3, 10		6
						β-Lactoglobulin	Endoprotease Lys-C	5		6
						WPC (79% protein)	Papain	3	TH, > 1kDa	14
						WPI	Rhozyme 41	0-5		17
1/time for 40 ml foam	WPI	Chymotrypsin	4-8	< 10 kDa	7	WPI	Chymotrypsin	3	< 10 kDa	7
		Trypsin, Chymotrypsin, Alcalase, Neutrase	3-8	> 10kDa			Alcalase, Neutrase, Trypsin	3-8	< 10 kDa	
foam volume	β-Lactoglobulin	Plasmin	4	< 2kDa	12	β-Lactoglobulin	Trypsin, Plasmin, *Staph aur*. V8	1, 2, 4		15
						β-Lactoglobulin	Plasmin	4	7-14 kDa	12

Measure	Protein	Enzyme	DH (%)	MW fraction	Ref [a]	Protein	Enzyme	DH	MW fraction	Ref
	DECREASED EMULSION STABILITY					*INCREASED/ SIMILAR EMULSION STABILITY*				
Time to reduce turbidity by 50%						Casein	Alcalase, chymotrypsin, papain	?, low		4
Centrifugation 'drainage'	Acid casein	Trypsin, Rhozyme, Chymotrypsin,	2-5		13					
	WPC (sweet whey)	Trypsin	?		8	WPC (sweet whey)	Chymotrypsin	?		8
EAI heating after 20-24 hr	Casein	*Staph. aur.* V8	2, 7		2	WPC 80	Trypsin	2.5, 4, 5		9
	Casein	Trypsin	4, 8, 10		9	WPC (79% protein)	Papain	3	TH, >1 kDa	14
Tubidity 24 hr	β-Lactoglobulin	*Staph. aur.* V8	2, 4		15	β-Lactoglobulin	*Staph. aur.* V8 Trypsin, Plasmin	1 1, 2, 4		15
	β-Lactoglobulin	Plasmin	4	< 2kDa	12	β-Lactoglobulin	Plasmin	4	7-14 kDa	12
Time point?						β-Lactoglobulin	End.Prot. Arg-C End.Prot. Glu-C/ Lys-C Trypsin	2 5 3, 10		6
Emulsion rating	WPI	Trypsin, Chymotrypsin, Alcalase, Neutrase	3-8	< 10kDa	7	WPI	Alcalase	3-8 6-8	> 10kDa TH	7
	WPI	Alcalase	3-4	TH	7	WPI	Trypsin, Chymotrypsin	3-8	TH, >10 kDa	7
	WPI	Neutrase	3-4	TH, > 10kDa	7	WPI	Neutrase	6-8	TH, >10 kDa	7
Particle size	β-Lactoglobulin	Trypsin	?		11					
	DECREASED FOAM STABILITY					*INCREASED/ SIMILAR FOAM STABILITY*				
Drainage	Na caseinate	Protamex	9, 15		1	Na caseinate	Protamex	0.5-1		1
	WPI (95% protein)	Pepsin, Acid fungal prot	~3	<, >10 kDa	16	WPI (95% protein)	Alcalase, chymotrypsin	~3	<, >10 kDa	16
	WPI (95% protein)	Trypsin	~3	> 10 kDa	16	WPI (95% protein)	Trypsin	~3	< 10 kDa	16
	WPI	Rhozyme 41	0-5		17	WPC (79% protein)	Papain	3	TH	14
	WPC (79% protein)	Papain	3	> 1kDa	14	β-Lactoglobulin	Endoprotease Lys-C	5		6
	β-Lactoglobulin	Endoprotease Arg-C	2		6					
	β-Lactoglobulin	Endoprotease Glu-C	7		6					
	β-Lactoglobulin	Trypsin	3		6					
Foam decay	WPI	Chymotrypsin, Alcalase, Neutrase	3-8	<, > 10kDa	7	WPI	Trypsin	4-8	< 10 kDa	7
	WPI	Typsin	3 8	< 10 kD > 10kDa	7					
Foam volume decrease	β-Lactoglobulin	Plasmin	4	< 2kDa	12	β-Lactoglobulin	Trypsin, Plasmin, *Staph. aur.* V8	1, 2, 4		15
						β-Lactoglobulin	Plasmin	4	7-14 kDa	12
	Casein	Chymotrypsin, Papain	?, low		4	Casein	Alcalase	?, low		4

[a] References: 1 (Slattery & Fitzgerald, 1998), 2 (Chobert *et al.*, 1988b), 3 (Svenning *et al.*, 1993), 4 (Ludwig *et al.*, 1995), 5 (Mahmoud *et al.*, 1992), 6 (Vojdani & Whitaker, 1994), 7 (Mutilangi *et al.*, 1996), 8 (Haque, 1990), 9 (Chobert *et al.*, 1988a), 10 (Kuehler & Stine, 1974), 11 (Agboola & Dalgleish, 1996), 12 (Caessens *et al.*, 1999a), 13 (Haque & Mozaffar, 1992), 14 (Lieske & Konrad, 1996), 15 (Caessens *et al.*, 1999b), 16 (Althouse *et al.*, 1995), 17 (Britten *et al.*, 1994), 18 (Turgeon *et al.*, 1992). [b] *Staphylococcus aureus.* [c] DH unknown. [d] Total hydrolysate. [e] Enzyme to substrate ratio, [f] Ionic strength. [g] Fractions were obtained from ion and/or hydrophobic interaction chromatography.

Differences in foam-stabilising behaviour of the permeate relative to the retentate fraction depended on the enzyme used for hydrolysis (Althouse et al., 1995). According to a study with a papain hydrolysate of whey protein, fractionated with a 1 kDa cut-off membrane, it was shown that the low-MW fraction (<1kDa) was essential for foam stabilisation, though for other emulsion and foam properties, no contribution of this fraction was detected (Lieske & Konrad, 1996). Finally, for a plasmin hydrolysate of β-lactoglobulin it was shown that the fraction containing peptides <2 kDa was not able to stabilise the foam, whereas the hydrolysate fraction containing larger peptides did form a stable foam (Caessens et al., 1999a).

From the above discussed literature reports and from Table 4 it can be concluded that for emulsion stability and emulsion-forming ability peptides with relative high molecular weights are important, while for foams low molecular weight peptides may also contribute to the stability. For foam-forming ability the lower molecular weight fraction also seems to contribute to the formation process, since the permeate fractions do form high foam levels. Although a general idea about the peptides important for the techno-functional behaviour is obtained from these studies, only a few molecular weight fractions have been studied which solely give an estimation of the molecular weights that are important. Most of the studies reporting on techno-functional properties of hydrolysates only give the DH as an indicator for the hydrolysate, but as already discussed, this parameter is just a very rough indicator for the hydrolysate. An overall correlation between DH and techno-functional properties of hydrolysates cannot be deduced from the results reported in literature.

CORRELATION BETWEEN VARIOUS PROPERTIES OF HYDROLYSATES

As described before, the peptide composition of a protein hydrolysate depends on the protein and enzymes used for hydrolysis as well as on the hydrolysis process conditions. Hydrolysates can be subsequently characterised according to several molecular characterisation methods, which reflect the molecular properties of the hydrolysates. Moreover, functional properties of hydrolysates will differ from that of the original protein and will depend on the peptide compositions of hydrolysates. The interactions between protein source, hydrolysis conditions and hydrolysate characteristics are schematically depicted in Figure 6. Little is known about the precise correlations between the hydrolysis conditions and the hydrolysate properties, or about the correlations between molecular and functional properties.

Until now, relations between molecular and functional properties have been studied using fractions with specific molecular properties, prepared from one or a few hydrolysates. This approach is based on hypotheses that peptides with particular molecular properties will exhibit a specific functional behaviour. However, fractionation is often performed rather arbitrarily. Moreover, with this approach only one molecular property can be studied at the time.

With new analytical and computational techniques it has become possible to analyse a multitude of data simultaneously, which allows for investigating correlations between several hydrolysate characteristics. In this approach data are obtained without previous assumptions

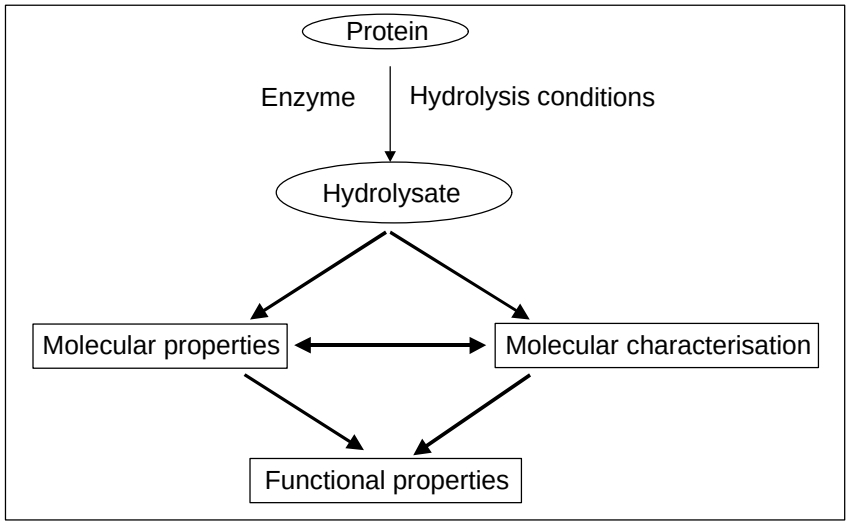

Figure 6: Relation between hydrolysis process, hydrolysis characteristics and detection methods.

on possible correlations between product characteristics. As these techniques may handle complex relations, simplification of samples beforehand, to deal with the problem in a comprehensive way, is no longer needed.
In the present study, three multivariate data analysis techniques were used, which are shortly introduced in the next paragraph.

Multivariate data analysis

Multivariate data analysis techniques are statistical methods applied to extract meaningful information out of large data sets (Dillon & Goldstein, 1984). Three multivariate data analysis techniques were applied in the present study:

- Principal Component Analysis (PCA), to study similarities and differences between samples.
- Regression analysis with highly correlated data, using the Partial Least Squares (PLS) method to study correlations between diverse molecular characterisation methods and between molecular characteristics and functional properties.
- Regression analysis with independent regression variables to optimise hydrolysis process conditions. The settings for the studied hydrolysis parameters were defined using experimental design and results were analysed with Response Surface Methodology (RSM).

Principal Component Analysis (PCA)

As described before, hydrolysates can be characterised by various biochemical and functional properties (hydrolysate parameters). Each hydrolysate is described by a unique set of values for all measured parameters, which allows for discrimination between hydrolysates. The measured parameters may be correlated to each other, implying that they contain partly overlapping information. Therefore, the differences between samples (hydrolysates) are described more efficiently (*i.e.* with less parameters) if only independent variables are used, each containing unique information. In PCA, these new independent variables are calculated

from the original parameters (Dillon & Goldstein, 1984). The method of calculation of such new variables is schematically depicted in Figure 7 for a simple example with two parameters (Jolliffe, 1986).

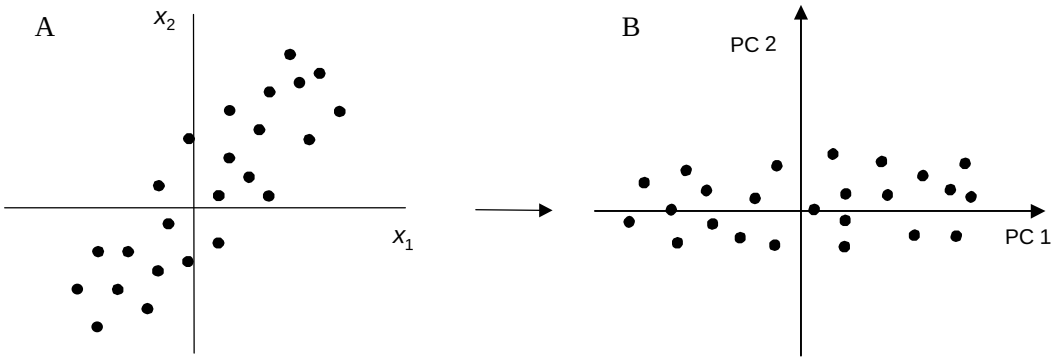

Figure 7: Schematic representation of the calculation of principal components in case of two variables, x_1 and x_2.

In Figure 7A, data points (samples) are described by combinations of values for x_1 and x_2. From this plot it appears that the x_1- and x_2-variables are highly correlated: an increase in x_1 is related to an increase in x_2. The variation between the data points can be described more efficiently by a new vector, which is calculated in the direction of the largest variation (Figure 7B). The new vector, the first Principal Component (PC 1), explains more variation in the data points than does either the x_1- or x_2-variable. The first PC does not explain all variation in the data. Therefore, a second PC is calculated, orthogonal to the first PC, which describes most of the remaining variation (PC 2). In PCA, by definition, the first principal component describes most of the variation between the samples, the second component describes most of the remaining variation and so on (Dillon & Goldstein, 1984; Jolliffe, 1986).
The new vectors are linear combinations of the original variables, which is described by equation 5 for the example in Figure 7.

$$PC\ 1 = p_{11}x_1 + p_{12}x_2 \quad \text{and} \quad PC\ 2 = p_{21}x_1 + p_{22}x_2 \qquad [5]$$

The original variables are related to the new vectors by 'loadings' (p_{11}, p_{12}, p_{21}, p_{22}). A high loading of a parameter on the vector implies that the variation in that parameter is explained to a large extent by that vector. In case two parameters have a high loading on the same vector, the parameters are positively correlated to each other (Dillon & Goldstein, 1984). Similarly to the original variables, the samples are transformed to co-ordinates on the new variables. Similar samples will appear in the same area of the newly defined variable space.

Regression analysis (PLS)

Regression analysis is used for determination of a relation between a set of, for example easy-to-measure, predictive variables (regressors) and a response variable of interest. With regression models responses for new variable settings can be predicted and insight about relations between regressors and response variables can be obtained. If regression analysis is

performed with correlated variables, the individual effects of the regressors on the response variable can not be interpreted, due to possible covariance between regression coefficients (Dillon & Goldstein, 1984). However, if the data are first transformed to independent principal components, these components can be used for regression analysis, allowing for an unambiguous interpretation of the effect of the regressors on the response parameter. If the data set is first decomposed to a set of new PC's and these PC's are used for regression, the method is called Principal Component Regression (PCR) (Montgomery et al., 2001). In this method the response parameter is not considered for calculation of the new PC's. Therefore, it might occur that there is no correlation between the first PC's and the response parameters, and higher order PC's are needed to make a PC regression model. A calculation method to circumvent this problem is PLS (Partial Least Squares) regression. In this regression model, during calculation of the new vectors (PC's), the response parameter is taken into account (Jolliffe, 1986). This results in a model where the first PC (usually) explains most variation in the response parameter. In the present study this method was used for regression analysis with correlated data.

Experimental design and Response Surface Methodology (RSM)

The above-described methods are generally used with arbitrarily chosen samples and variables. In order to describe samples, a large set of variables is measured and subsequently correlations are studied. A different situation occurs when a set of predefined variables, thought to influence the response parameter of interest, is varied in a structural manner in order to study the effects of the variables on the response variable. This is for example the case in process optimation studies. In these studies (process) variables are mutually independent and the effect of these variables on the response variable can be studied using linear regression analysis.

If all variables have to be optimised consecutively many experiments are needed, especially when variables have interactive effects. Therefore, experimental designs are developed for efficient experimentation. One example of an experimental design is the Central Composite Rotatable Design (CCRD) as described by Cochran and Cox (Cochran & Cox, 1957). In this design five levels are defined for each variable coded as 0,1,-1,α, and -α. The value of α depends on the number of variables studied. For three variables the experimental space is represented in Figure 8.

Three types of experiments are performed: centre experiments, with all variables set at level 0, cube experiments, which are combinations of –1 and 1 settings, and star experiments, in which one variable is set at an extreme level (α or -α) and the other variables are set at level 0. The advantage of a rotatable design is the constant variance of a response variable at a fixed distance from the centre point. The central composite design is one of the most important designs used for the study of quadratic response surfaces (Myers & Montgomery, 1995).

From the performed experiments a response surface model can be calculated in which the output variables are a function of the *x*-variables: $y = f(x_1, x_2, x_3, \ldots x_k) + e$. Most commonly, quadratic models are used, considering linear as well as quadratic and two-factor interaction effects. With the regression parameters two-dimensional response surfaces, reflecting the

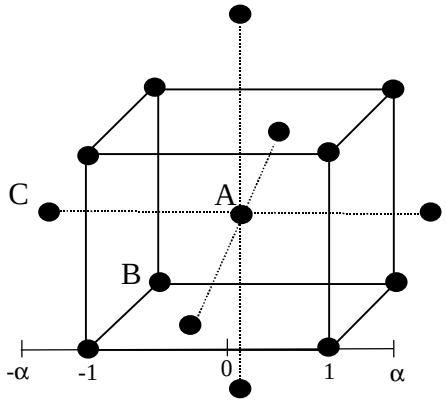

Figure 8: Schematic representation of a central composite rotatable design with five levels. Three types of experiments are defined: centre (A), cube (B) and star (C) experiments.

effect of variation in two process conditions on one response variable, can be constructed. With these response surfaces optimal variable conditions can be estimated (Myers & Montgomery, 1995).

AIM OF THE STUDY

Various studies have been published describing functional parameters of protein hydrolysates. Generally, only a few enzyme/protein systems are studied in each report and far most studies only mention DH as a molecular property of hydrolysates (Table 2). Proposed structure-function relations for peptides are based on studies performed with pure peptides (Lee et al., 1987; Caessens et al., 1999b; Caessens et al., 1999c; Poon et al., 2001) or performed with fractions of hydrolysates obtained from chromatography or ultrafiltration (Turgeon et al., 1991b; Huang et al., 1996; Mutilangi et al., 1996; Caessens et al., 1999a). Whether these findings are also valid for complete hydrolysates prepared for commercial purposes is not clear. Moreover, studies on functional properties of hydrolysates use different methods to measure the functional properties, which hinders comparison of the results. Therefore, conclusions about general factors relevant for specific functional parameters can not be drawn from literature.

The present study differs from reported studies by its holistic approach. Analytical characteristics and functional properties of a large set of hydrolysates are determined and subsequently analysed with multivariate data analysis. This approach was initiated in order to improve the understanding of correlations between molecular and functional properties of hydrolysates, being valid for numerous hydrolysates. Moreover, the ability of several analytical methods to be used as hydrolysate fingerprints was investigated, as hydrolysate fingerprints are in potential useful as indicators for several molecular and functional properties.

OUTLINE OF THE THESIS

In the present study hydrolysates of sodium caseinate and whey protein concentrate were prepared with eleven different commercially available food-grade enzymes. Of each protein/enzyme combination three hydrolysates were prepared, which were characterised by *e.g.* their degrees of hydrolysis, size exclusion chromatograms, reversed phase chromatograms and fourier transform infrared spectra (FTIR). Several techno-functional properties of these hydrolysates were determined, *i.e.* bitterness, solubility, emulsion- and foam properties as well as one bio-functional property, *i.e.* ACE-inhibiting activity.

Separation of hydrolysates on size exclusion and reversed phase materials is partly due to the same molecular properties. In Chapter 2 the characterisation of hydrolysates by these two methods is compared and their relation is studied using multivariate analysis. Moreover, the influence of the amino acid composition of peptides on their retention time on RPC is also studied. A suggestion is made for calculation of retention time coefficients for peptide amino acids in hydrolysates of various protein sources.

Emulsion-forming ability and stability was studied for part of the whey and casein hydrolysates (Chapter 3). The large number of hydrolysates tested permitted for an overall view about biochemical factors that contribute to emulsion properties. The relation between emulsion stability and emulsion-forming ability was investigated and both emulsion properties were regarded in relation with the molecular weight distributions of hydrolysates.

Foam properties of hydrolysates were studied for the same hydrolysates as the emulsion properties, also in relation to molecular weight distribution of the hydrolysates (Chapter 4). Correlations between MWD and foam- and emulsion properties were investigated using multivariate regression techniques.

Apart from hydrolysate characterisation by size exclusion and reversed phase chromatography, hydrolysates were also characterised using Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy is based on infrared adsorption by molecular bonds. FTIR characterisation of complex mixtures does not reveal information on individual compounds, but can be used as a fingerprint method. The use of FTIR for characterisation of hydrolysates and the ability to predict functional characteristics from FTIR spectra is described in Chapter 5.

As described before, ACE inhibition is caused by a variety of peptides, formed from various protein sources and with various enzymes at divers process conditions. The combination of peptides in a hydrolysate determines its final ACE inhibiting ability. Peptide composition depends on the choice of hydrolysis conditions. The effect of changing hydrolysis conditions on ACE inhibitory activity of pancreatic whey protein hydrolysates was investigated. Hydrolysis process was optimised towards maximum ACE-inhibiting activity, using experimental design and response surface methodology (Chapter 6).

Finally, correlations between functional and molecular properties not treated in one of the chapters 2-6 are presented in Chapter 7. Also the possibility to use LC/MS analysis for identification of peptides responsible for a certain functional property of interest is described in Chapter 7. Finally a general conclusion on the work presented in this thesis is given.

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CHAPTER 2

Reversed phase and size exclusion chromatography of milk protein hydrolysates: Relation between elution from reversed phase column and apparent molecular weight distribution

ABSTRACT

Hydrolysates of casein and whey protein were analysed with size exclusion and reversed phase chromatography. Hydrolysate elution patterns obtained from the two chromatographic techniques were correlated. The apparent molecular weight distribution of the peptide mixtures could be predicted accurately from their reversed phase chromatograms, using partial least squares regression. Reversed phase profiles of specific molecular weight fractions overlapped considerably, indicating that the relation between reversed phase chromatography and molecular weight distribution is complex.

Retention times of defined casein peptides on a reversed phase column were predicted by summing retention time coefficients of the constituent amino acids. The coefficients were calculated by linear regression from a data set of peptide sequences and retention times obtained from LC/MS analysis of casein hydrolysates. The newly calculated retention time coefficients resulted in better prediction of retention times of casein peptides than did prediction with coefficients from literature.

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INTRODUCTION

Hydrolysis of food proteins is performed for various purposes, varying from reduction of allergenic properties to changing functional properties like solubility, gelation, emulsifying and foaming properties. To characterise the products that are made under various conditions, analytical methods like size exclusion chromatography (SEC) and reversed phase chromatography (RPC) are commonly used.

Separation of components using SEC is principally based on differences in the hydrodynamic volume of molecules, which depends on size and conformation. Several articles have been published on the determination of the molecular weight distribution (MWD) of peptides in hydrolysates, reporting also non-ideal retention of peptides due to electrostatic and hydrophobic interactions (Visser et al., 1992; Silvestre et al., 1994; Fujinari & Manes, 1997; Smyth & Fitzgerald, 1997; Tossavainen et al., 1997).

Reversed phase chromatography is primarily based on differences in hydrophobicity. In case of small peptides (less than 15 residues) retention time seems to depend only on amino acid composition, while retention time of larger peptides is also influenced by other effects like peptide length (Mant et al., 1988). The correlation between amino acid composition and RPC retention time of small peptides was demonstrated by predicting the peptide retention time on basis of retention time coefficients of the constituent amino acids, which represent the contribution of each amino acid to the retention of peptides. Peptide retention time is predicted by summing the coefficients of the constituent amino acids and constants depending on column characteristics. Several researchers computed the coefficients by linear regression using retention behaviour of peptides with known sequences (Meek, 1980; Wilson et al., 1981; Sasagawa et al., 1984; Wilce et al., 1993). In another approach Guo and coworkers (1986a) used series synthetic peptides differing in only two amino acids to calculate retention time coefficients. Both methods resulted in rather good prediction of peptide retention times for peptides smaller than 15 residues (Guo et al., 1986b; Hearn et al., 1988).

In case of longer peptides and proteins, however, other factors like conformational effects and molecular weight influence retention behaviour (Pearson et al., 1984). Prediction of retention time of proteins and peptides between 3.5 and 32 kDa on basis of amino acid retention time coefficients indeed did not work (Mant et al., 1989). Chabanet and Yvon (1992) proposed a model in which the retention time coefficient of each amino acid depends on the length of the peptide, showing rather good correlation for peptides up to 50 residues.

In literature, amino acid sequences and lengths of single peptides are used to describe the elution of peptides on RPC columns. In this study we are interested in reversed phase chromatography of complete hydrolysates, which are mixtures of peptides with unknown sequence, in relation with their molecular weight distribution. As RPC and SEC chromatograms contain a large amount of information, multivariate data analysis was used for processing the results. Additionally, for prediction of RPC retention time of small casein peptides, a new set of retention time coefficients was calculated from retention times of 43 casein peptides and compared to retention time coefficients from literature. The predicting ability of the various sets of retention time coefficients was compared using another set of casein peptides.

MATERIALS AND METHODS

Materials

Sodium caseinate (89% protein based on dry weight) was obtained from DMV International (Veghel, The Netherlands), whey protein (WPC 60, 60% protein on dry weight) from Milei GmbH (Stuttgart, Germany). Acetonitrile was purchased from Biosolve, TFA from Merck. Milli-Q water was prepared with a Millipore system, water was filtered over a 0.22 μm filter (Millipak).

An HPLC system was used with system controller (LC-10A), pump (LC10-Ai), injector (SIL-10Ai), column oven (CTO-10AC) and UV detector (SPD-10Avp), all from Shimadzu. A P-500 pump (Pharmacia Biotech) was used for solvent delivery for SEC analysis.

Production of hydrolysates

Casein and whey protein were hydrolysed with eleven different enzymes, under conditions as given in Table 1. Enzymes were obtained from Novo Nordisk (Pem, Flavourzyme, Alcalase), Biocatalyst (Promod 184, Promod 258, Pepsin), Genencor (Protex 6L), Amano (NewlaseF), Röhm (Corolase PP, Corolase L10) and Valley Research (Validase FP).

Table 1: Hydrolysis conditions.

Enzyme	pH	Temp (°C)	E/S[a] (%)	
			Casein (Cn[b])	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] 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.

Protein suspensions or solutions, 800 ml 5% (w/w) protein, were hydrolysed in a pH-Stat set-up (STAT-Titrino 718, Metrohm). Whey protein suspensions were held at 90 °C for 15 min prior to enzymatic digestion to improve digestibility; casein solutions were not pre-treated. In preliminary experiments the maximum degree of hydrolysis (measured as the maximum amount of NaOH added) was determined for each enzyme/substrate combination. Enzyme concentration (adjusted to a concentration high enough to reach maximum hydrolysis within 3 hours of hydrolysis) and sample times in the final hydrolysis experiments were based on these preliminary results. Samples (200 ml) were taken at 1/3, 2/3 and the maximum degree of hydrolysis. Enzymes were inactivated by heating for 15 min at 90 °C. The hydrolysates were

centrifuged (30 min, 3000x*g*, 20 °C) at the pH of hydrolysis. Supernatants were freeze-dried. Supernatants were used for chromatography. Sample codes are sequentially composed of 2 digits for protein source, 3 digits representing the enzyme used and 2 digits encoding the degree of hydrolysis reached, for example CnNwf06: casein, Newlase F, DH=6%. Protein and enzyme codes are given in Table 1.

Size exclusion Chromatography

Analytical SEC

Size exclusion chromatography was 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 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 (0.45 µm cellulose acetate filter). The eluate was monitored at 200 nm.

Preparative SEC

Fractionation of hydrolysates was performed with a Biopilot system (Pharmacia) on preparative Superdex 30 (60 x 10 cm) column (Pharmacia). Hydrolysate (30 ml, 0.1 g/ml) was dissolved in eluent, undissolved particles were removed by centrifugation (30 min, 10.000x*g*, 4°C). The mobile phase consisted of 66 mM NH_4HCO_3 and was run at a flow rate of 40 ml/min. The eluate was monitored at 220 nm. Fractions of 350 ml were collected and desalted on a reversed phase column (8x3.5cm, Source 15, Pharmacia Biotech). Samples were loaded onto the column in 100 % eluent A (Milli-Q, 0.016% TFA). Next, peptides were eluted by changing to 100% eluent B (80% acetonitrile, 0.05% TFA). Acetonitrile was evaporated and samples were subsequently freeze-dried.

Reversed Phase Chromatography

Reversed phase analysis was performed on a Hi-Pore Reversed Phase Column RP 318 (250 x 4.6 mm) (BioRad) using a Shimadzu HPLC system. A sample (30 mg/ml) was dissolved in buffer A and remaining particles were removed by filtration over 0.45 µm cellulose acetate filter, followed by injection of 20 µl on the column. Analysis was performed at 25 °C with a flow rate of 1 ml/min, using a linear acetonitrile gradient, starting with 2.5 min 100% buffer A (water, 0.13% TFA), followed by a linear gradient to 40% buffer B (100% acetonitrile, 0.13% TFA) in 56 min. After analysis the column was equilibrated for 12.5 min with buffer A. Since the increasing amount of acetonitrile will result in increase of baseline absorption if detection of 200 nm is used, the eluate was monitored at 220 nm.

LC/MS

Peptide sequences were determined on a LC/MS system. Peptide mixtures (20 µl, ± 1 mg/ml) were separated on a Supersphere RP18E (125 x 2.0 mm) column (Bischoff) using a HPLC (Thermo Separation Products P2000) equipped with UV meter (UV1000) and autosampler (AS3000). Peptides were eluted with acetonitrile gradient; 2 min 100% eluent A (Milli-Q, 0.1% TFA), followed by a linear gradient from 0 – 100% eluent B (95% acetonitrile, 0.1% TFA) in 30 min. Subsequently 100% eluent B was pumped over column for 8 min, followed by column re-equilibration with eluent A for 9 min. The flow rate was 0.3 ml/min. The mass spectrum was recorded with an Electrospray Ionisation (ESI) octapole mass spectrometer with iontrap (LCQ MS, Finnigan MAT) operating in the positive mode. Mass spectra existed of a full mass scan, followed by a zoom scan and a MS/MS scan of the most intense ion. These scans were used to identify peptide sequences with SEQUEST search program (Finnigan).

Multivariate data-analysis

SE-HPLC and RP-HPLC curve data were analysed with multivariate data analysis (The Unscrambler®, CAMO) using principal component analysis. Chromatograms were normalised (mean normalisation) before analysis, to minimise concentration effects. In principal component analysis a new set of independent variables describing the samples is created. Typically the number of new variables is less than the number of original variables. The first principal component (PC) describes the most important differences between the samples (Jolliffe, 1986).

Regression analysis was performed with RPC profiles as *x*-variables and arbitrarily chosen apparent molecular weight intervals of the SEC profiles (>5 kDa, 4-5, 3-4, 2-3, 1-2, 0.5-1 and < 0.5 kDa, based on the peptides calibration set) as *y*-variables. Regression was performed with PLS (Partial Least Squares) method, with full cross validation. In PLS, principal components are constructed from the predictor variables (*x*-variables), followed by a rotation of these PC's to reach a better correlation with the *y*-variables (Jolliffe, 1986).

RESULTS AND DISCUSSION

In Figure 1, typical chromatograms of SEC and RPC analysis (measured on the Biorad column) of three casein hydrolysates are given. Samples were representative for both casein and whey samples. From the SEC chromatograms (Figure 1a) it can be seen that sample CnNwf06 contains the highest amount of large peptides, while sample CnAlc19 contains mainly small peptides and sample CnPem09 is composed of peptides with moderate molecular weight. Separation of these samples on RPC (Figure 1b) shows that peptides in sample CnNwf06 elute mainly at the end of the chromatogram, that peptides in the CnAlc19 sample generally elute faster, while peptides in sample CnPem09 elute over the entire range. These differences indicate a possible relationship between apparent molecular weight of peptides and retention on RPC column.

It should be noted that the chromatographic elution patterns were recorded at 200 and 220 nm, for respectively SEC and RPC. These wavelengths are generally used for these

chromatographic methods (Meek, 1980; Chabanet & Yvon, 1992; Smyth & Fitzgerald, 1998; Pouliot et al., 1999). Absorption at these wavelengths is mainly caused by peptide bonds (Woods & O'Bar, 1970; Becklin & Desiderio, 1995), which implies that the amount of small peptides is underestimated. However, it is not possible to use a correction factor, since the exact composition of the low molecular weight fraction is not known. Other simple methods for detection of peptides are not available; peptide bonds absorb from 185 to 220 nm, whereas at higher wavelengths only aromatic amino acids are detected. Therefore, despite its imperfection, absorption at 200 and 220 nm were used to determine to protein/peptide concentration.

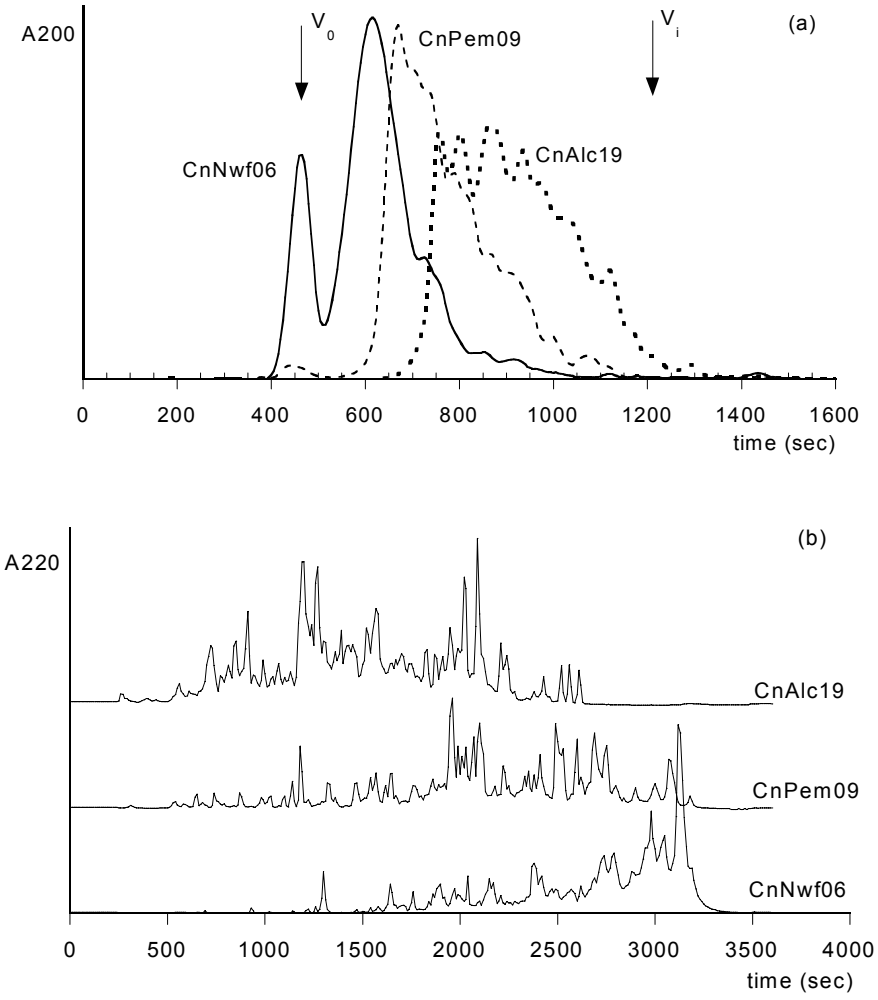

Figure 1: Chromatographic plots of three casein hydrolysates (CnNwf06, CnPem09, and CnAlc19). a) size exclusion profiles, b) reversed phase profiles, measured on the Biorad column (see materials and methods section).

Statistical analysis of data

In this study, reversed phase and size exclusion chromatograms of 66 hydrolysates were examined. Since interpretation of all these data is complex, multivariate data analysis was used, allowing for comparison of all chromatograms at the same time. With principal

component analysis data were reviewed to investigate the homogeneity of the data set. Knowledge about existence of sample groups or outliers is important before starting with regression analysis. With regression analysis, the relation between both chromatographic methods was studied.

Principal component analysis of SE-HPLC and RP-HPLC chromatograms

The results of the principal component analysis of all SEC and all RPC data are represented by score plots (Figure 2a, 2b), which show the position of the hydrolysates on the first two principal components (PC's). In case of the SEC data (Figure 2a), PC 1 explains 60% of the variance between samples and PC 2 explains 22% of the remaining variance. Whey and casein samples are spread over the entire plot and are not clustered into groups, implying that the SEC profiles do not represent information about the protein source. Analysis of RPC profiles does reveal grouping of samples (Figure 2b). Three sample sets can be distinguished: a set

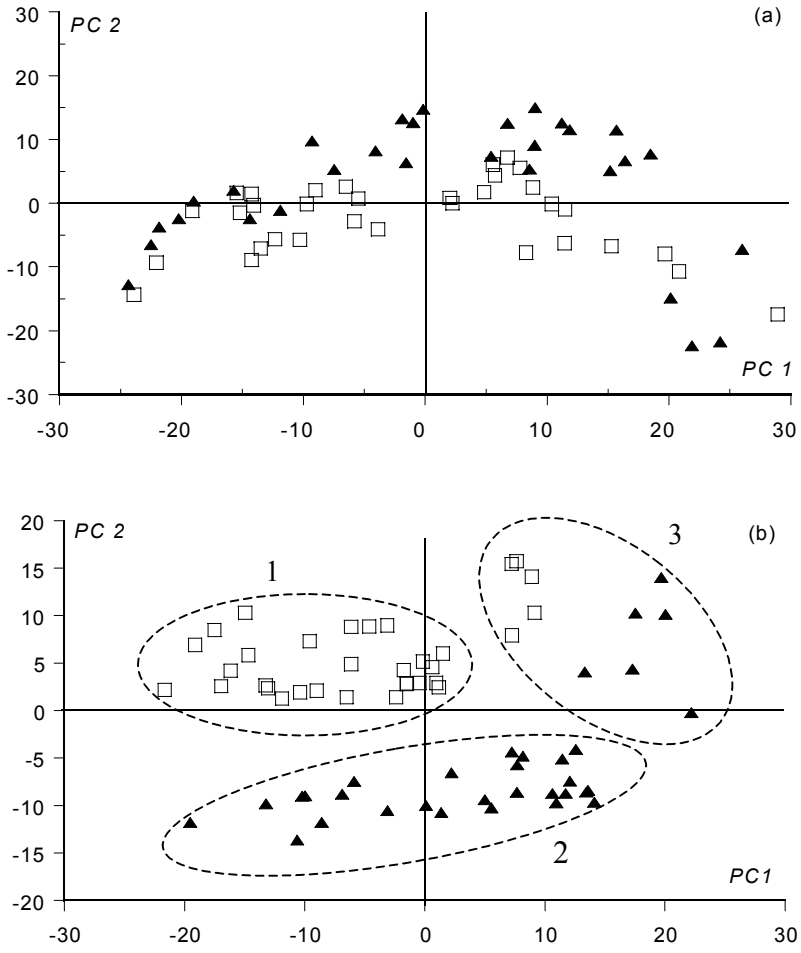

Figure 2: Principal component plots of whey (□) and casein (▲) hydrolysates based on size exclusion (a) and reversed phase chromatography (b).

containing solely whey hydrolysates (nr 1), a set containing solely casein hydrolysates (nr 2) and the third group containing both casein and whey hydrolysates (nr 3). Classification of samples revealed that the three groups were indeed statistically different (p=0.01). The existence of three sample groups is important for further regression analysis.

Relation between reversed phase profiles and molecular weight distribution

Figure 1 shows that samples with a relative high proportion of large peptides eluted later from RPC column than samples containing mainly small peptides (CnAlc19 versus CnNfw06). Studies aimed at the prediction of retention time of defined peptides on RPC columns already showed that peptide length influences retention, especially if peptides were larger than 15 amino acids (Guo et al., 1986b; Mant et al., 1988; Mant et al., 1989; Chabanet & Yvon, 1992). To investigate the relation between RPC elution and molecular weight distribution of total hydrolysates, regression analysis was performed with RPC profiles as predictors and molecular weight fractions as *y*-variables. Regression was performed with three sample sets: only casein samples, only whey samples and one set containing all samples. As seen in Figure 2b the six casein samples from group 3 differ from the other casein samples. These samples contain a large amount of peptides having high retention times on the RPC column. Regression analysis of all 33 casein samples show that if these six samples are included, prediction of molecular weight distribution is less accurate compared to a model without these samples. The overall correlation coefficient of the model is 0.79 for all casein samples and 0.86 for the casein model with 27 samples. The main difference in prediction is observed for the high molecular weight fractions. To obtain a model for casein hydrolysates with the highest accuracy, the six samples from group 3 were excluded, based on above described arguments. To predict molecular weight distribution on basis of RPC retention profiles of new casein hydrolysates, it should be assured that RPC profiles are similar to RPC chromatograms of samples belonging to group 2.

The five whey hydrolysates from group 3 did not influence the prediction of the molecular weight distribution of the whey hydrolysates and were included in the analyses.

The result of the prediction of the proportion of the molecular weight fraction 0.5–1 kDa of 33 whey and 27 casein hydrolysates based on their RPC profiles and molecular weight distribution is given in Figure 3, showing a good correlation between predicted and measured values. Prediction of other molecular weight fractions revealed similar results, as shown in Table 2, which displays parameters describing the regression line of the predicted versus measured values of all molecular weight fractions.

Data from Figure 3 and Table 2 show that prediction of molecular weight fractions based on RPC profiles is rather accurate. Correlation coefficients of measured versus predicted values for the molecular weight fractions were all larger than 0.8, except for the fraction 2-3 kDa in all models (R^2 = 0.55-0.72) and fraction 1-2 kDa in case of the model containing whey samples (R^2 = 0.74). All models result in good overall correlations, indicating that the relation between RPC profile and molecular weight distribution is not protein specific. The multivariate data analyses show that in case of peptide mixtures a clear correlation exists between elution on RPC column and apparent molecular weight distribution.

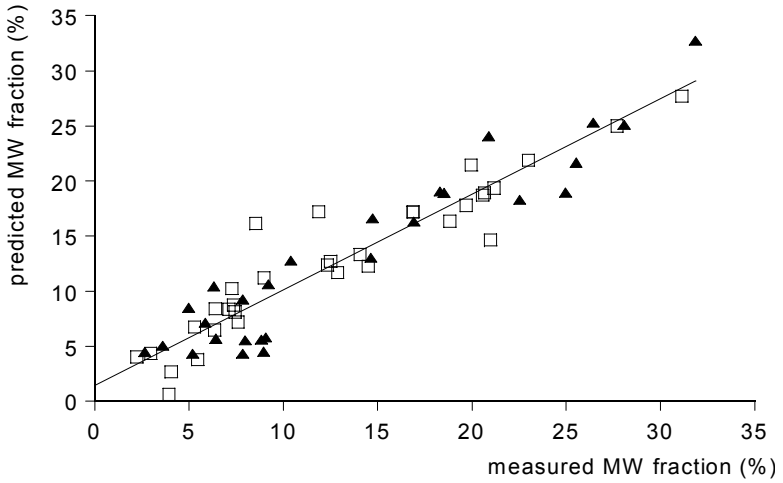

Figure 3: Prediction of the proportion of the molecular weight fraction 0.5-1 kDa of whey hydrolysates (□) and casein hydrolysates (▲). Predicted values (via RP-HPLC) are plotted versus measured (SE-HPLC) values. Prediction is based on the model with both whey and casein samples.

Table 2: Prediction of MW fractions based on RPC profiles; parameters describing the regression line of the predicted versus measured values.

	Casein (27 samples)			Whey (33 samples)			Casein and Whey (60 samples)		
R^2 of Model	0.86			0.88			0.81		
MW fraction (kDa)	Slope	R^2 [a]	SEP[b]	Slope	R^2	SEP	Slope	R^2	SEP
> 5	0.92	0.90	3.13	0.95	0.94	4.35	0.88	0.85	6.06
4-5	0.94	0.92	1.91	0.93	0.86	1.56	0.87	0.83	2.29
3-4	0.92	0.88	2.21	0.87	0.85	1.71	0.82	0.81	2.46
2-3	0.66	0.55	2.36	0.77	0.72	2.13	0.63	0.59	2.62
1-2	0.88	0.81	3.06	0.91	0.90	2.35	0.80	0.74	3.89
0.5-1	0.92	0.92	2.32	0.87	0.92	2.17	0.86	0.88	2.67
< 0.5	0.94	0.92	2.32	0.91	0.90	2.69	0.92	0.92	2.45

[a] R^2 = correlation coefficient (coefficient of determination).

[b] SEP= standard error of prediction.

Analysis of specific molecular weight fractions on reversed phase column

To investigate whether specific parts of the RPC chromatogram are related to specific molecular weight fractions, three hydrolysates (WcCPP12, CnP5815 and CnPem09) were fractionated into 18 fractions on a preparative Superdex-peptide size exclusion column (Figure 4a). The fractions were subsequently analysed on analytical SEC column and RPC column (measured on Biorad column). For three fractions the results are given in Figure 4.

The chromatographic analyses of the three fractions depicted in Figure 4 show that the peptide mixture containing small peptides (WcCPP12-C) elutes faster on RPC column (Figure 4c) than the mixture with large peptides (WcCPP12-A). However, the elution patterns overlap for a considerable part, since all fractions contain peptides eluting between 20 and 30 min.

Analyses of the other hydrolysate fractions show similar effects. Apparently, there is no direct relation between molecular weight fraction and retention on RPC column, although a general shift from low to high retention time with increasing peptide length is observed. The entire RPC chromatogram is needed to predict the size of a specific molecular weight fraction. With multivariate analysis using principal component analysis and regression analysis this complex relation can be described.

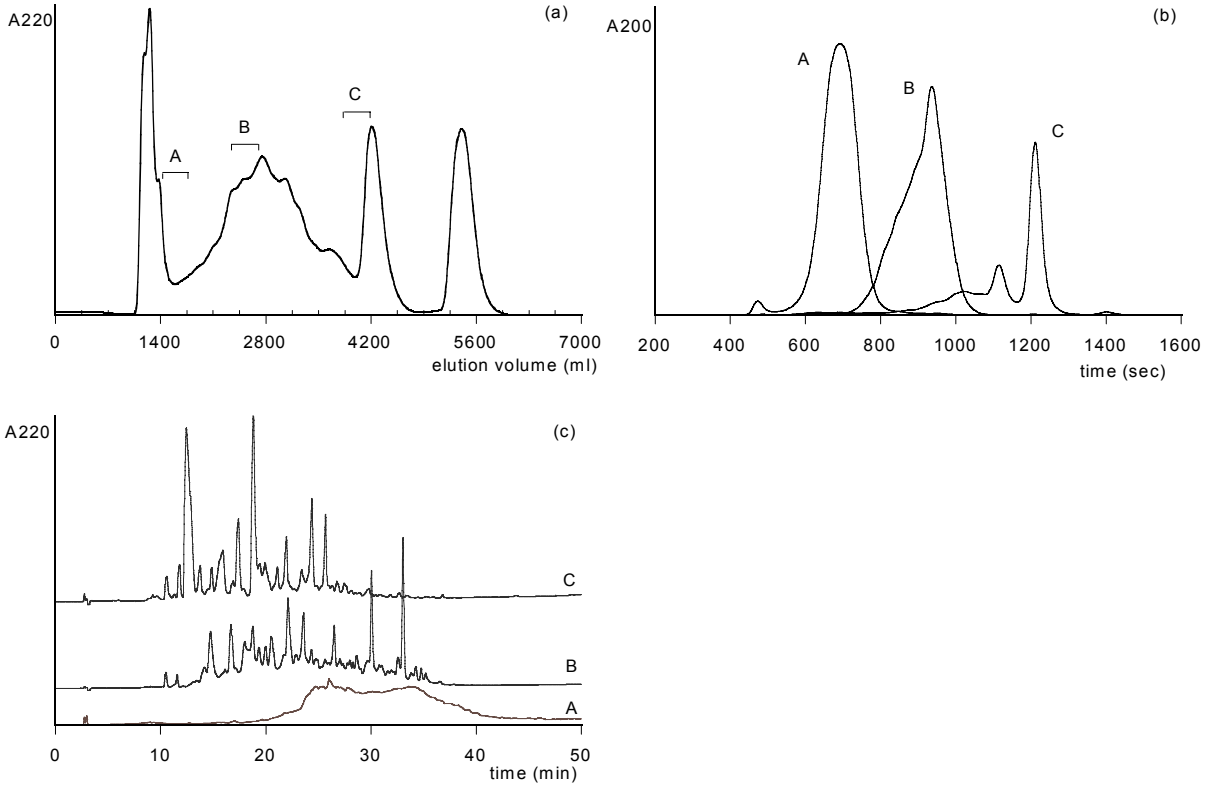

Figure 4: Separation of whey hydrolysate WcCPP12 on a preparative Superdex 30 column and characterisation of three fractions on SEC and RPC (measured on Biorad column). a: chromatogram of preparative size exclusion chromatography, b: analytical SEC of fractions A-C, c: RPC of fractions A-C.

The above shows a correlation between RPC retention times and molecular weight distribution, also for the peptide fraction <2 kDa (Table 2), which is in contradiction to published results describing that RPC retention times for peptides <15 residues only depend on hydrophobicity (Guo et al., 1986a).

In studies concerning prediction of retention times of peptides longer than 15 residues, the peptide length effect is taken into account in order to improve prediction of retention time of peptides, otherwise retention time of large peptides is overestimated. In the present study, the effect of peptide length on RPC retention is considered in another way, *i.e.* not on a one-peptide basis but in complete hydrolysates. As can been seen from Figure 1 and Figure 4, the chromatographic profiles of hydrolysates show an overall change from low to high retention

times if the proportion of large peptides in the hydrolysate increases. The correlation between RPC retention and peptide lengths is only revealed by use of statistical analysis.
The observed relation between the chromatographic techniques might also result from non-ideal separation on the size exclusion column (Visser et al., 1992; Lemieux et al., 1997; Tossavainen et al., 1997). However, the correlation coefficient of the molecular weight standards (MW 12,500 to 200 Da) to the regression line was 0.94, indicating minimal interactions between peptides and column material.

Retention time of defined peptides on reversed phase column

In the results presented above a correlation was described between RPC profiles and molecular weight distributions, also for molecular weight fractions smaller than 2 kDa. This seems to conflict with the theory that retention time of small peptides can be predicted purely on basis of amino acid composition according to the formula $Rt = \sum Rc + t_s + t_0$, with t_s and t_0 being constants depending on the RPC column and Rc being the retention time coefficient for each amino acid (Guo et al., 1986a). To verify if in our system peptide retention time can be predicted with retention time coefficients, hydrolysate fractions were analysed on LC/MS in order to relate amino acid composition to retention times on the LC/MS reversed phase column. Retention time coefficients can subsequently be calculated by linear regression (Meek, 1980; Wilson et al., 1981; Sasagawa et al., 1984; Wilce et al., 1993). In the present study 43 casein peptides (appendix) from casein hydrolysates were used to calculate new retention time coefficients. The prediction of retention times of the 43 peptides with the computed coefficients was reasonably good, the correlation coefficient was 0.86.
In Table 3, the retention time coefficients as calculated in the present study are given, as well as coefficients found by other researchers. The table reveals some remarkable differences between the retention time coefficients of the three models. Both the values of the coefficients and the ranking of the coefficients differ. Guo and coworkers (1986a) determined the retention time coefficients by use of synthetic model peptides, while for the calculation by

Table 3: Retention time coefficients calculated by linear regression with 43 casein peptides (appendix) compared with coefficients given by Guo et al. (1986a) and by Meek (1980).

Amino acids	casein model	Guo	Meek	Amino acids	casein model	Guo	Meek
G	-2.3	-0.2	-0.5	N	1.0	-0.6	-1.6
R	-1.7	-0.6	-4.5	A	0.8	2	-0.1
K	-1.1	-2.1	-3.2	L	1.4	8.1	10
T	-1.0	0.6	1.5	Y	1.5	4.5	8.2
D	-0.8	0.2	-2.8	H	1.5	-2.1	0.8
S	-0.7	-0.2	-3.7	V	1.8	5	3.3
Q	-0.4	0	-2.5	W	1.8	8.8	18.1
P	-0.3	2	8	I	2.2	7.4	11.8
E	-0.3	1.1	-7.5	F	2.6	8.1	13.9
M	0.7	5.5	7.1	C		2.6	-2.2

linear regression peptides from casein digests (this study) or pure peptides (Meek, 1980) were used. If peptides from digests are used some amino acids might be over-represented, which might influence the calculation. Moreover, as all these RPC analyses were performed at pH 2, the amino groups of the N-terminal amino acids are protonated, influencing retention time. In calculations of Guo et al. (1986a) and Meek (1980) a constant value was used to correct for the protonated amino group. However, Serada and coworkers (1993) reported that the effect of the protonated amino group depends on the N-terminal residue. This is not taken into account in the calculations of each of the three models.
The prediction power of the three sets of retention time coefficients of Table 3 was compared by prediction of the retention time of another 22 casein peptides (appendix), not used for building our linear regression model. The prediction of the new peptides was most adequate when the newly calculated coefficients were used (Figure 5). The prediction of retention times of the casein peptides with the coefficients reported in literature (Meek, 1980; Guo et al., 1986a) was not accurate.

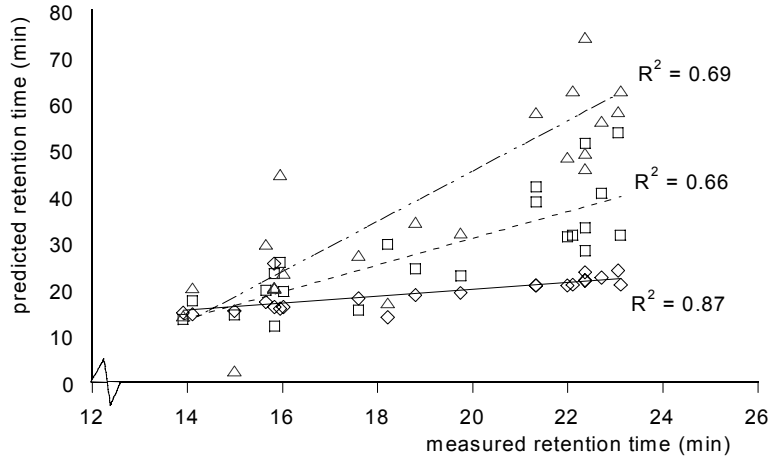

Figure 5: Prediction of retention time of 22 casein peptides on RPC column based on amino acid sequence. Retention time coefficients were used from the present study (◇), Guo et al. (□) or Meek (Δ).

The coefficients calculated from the casein peptides are possibly only applicable for prediction of peptides from the same protein source. To test if the 'casein retention time coefficients' can be used for the prediction of peptides from other protein sources, RPC retention times of 20 soy peptides were predicted. Retention time was also predicted with coefficients from literature (Table 3) and with newly calculated retention time coefficients from a set of another 40 soy peptides. These calculations showed that prediction with the new 'soy retention time coefficients' ($R^2 = 0.75$) was better than with the literature coefficients ($R^2 = 0.6$ and 0.55 for respectively Guo et al. and Meek) or with the 'casein retention coefficients' ($R^2 = 0.18$). As has been demonstrated clearly by these calculations, prediction of retention time of peptides from protein hydrolysates can be carried out most accurately with retention time coefficients obtained from a linear regression model composed of peptides from the same protein source. When universal coefficients are used a less optimal correlation between

measured and predicted values was found. In principle this non-universal coefficient approach is not more laborious than published methods. Analysis of some hydrolysates with LC/MS already results in a data set of peptide sequences and retention times large enough to calculate retention time coefficients.

CONCLUSIONS

Molecular weight distribution of hydrolysates could be predicted on basis of the reversed phase profiles of these hydrolysates. The prediction of the amount of peptides smaller than 2 kDa was also accurate, indicating that also in case of small peptides retention on RPC column is influenced by peptide length. For prediction of RPC retention time of small peptides (smaller than 15 residues) on basis of retention time coefficients, it is best to calculate the coefficients from a data set of retention times and amino acid compositions of peptides from the same protein source. With LC/MS these data sets can be easily obtained.

AKNOWLEDGEMENTS

The authors thank Gerrit de Vrij for his contribution to the chromatographic analyses.

Appendix: Peptide sequences used to calculate retention time coefficients (left part) and peptides used to compare predicted and measured retention times for three sets of retention time coefficients.

Casein peptides used for calculation of retention time coefficients	Retention times		Casein peptides used for prediction of retention time	Retention times			
	Measured	Predicted		Measured	Predicted		
	LC/MS	linear regression		LC/MS	casein model	Guo[a]	Meek[b]
KPWIQPKT	14.9	14.8	GPFPIIV	23.1	21.0	31.7	62.8
KKYKVPQLE	15.3	15.4	DELQDKIHPF	19.8	19.2	22.9	32.2
KKYKVPQL	15.2	15.6	SKVLPVPQ	15.7	17.2	19.8	29.7
YQEPVLGPVR	16.4	16.2	YPVEPFTE	18.8	18.7	24.4	34.4
SLPQNIPPLT	16.9	18.3	APFPEVF	22.4	21.9	28.3	46.0
YLEQLLR	17.5	16.9	VAPFPEVF	22.4	23.7	33.3	49.3
TQYTDAPSFSDIPNPIG	17.7	17.0	GYLEQ	13.9	15.0	13.5	14.2
KEMPFPKYPVEPFTE	17.9	18.9	RYL	15.8	16.2	12.0	20.2
LPLPLLQ	19.1	19.9	FRQF	17.6	18.0	15.6	27.3
KKYKVPQLE	15.7	15.4	FYPELFR	22.0	20.8	31.3	48.5
PPFLQPEVM	23.6	20.0	AIPPKKNQDKTEIPTINT	16.0	15.8	25.8	44.7
KKIEKF	16.1	16.1	IQKEDVPSE	15.0	15.3	14.5	2.4
QLLR	15.1	15.8	SQSKVLPVPQ	16.0	16.2	19.6	23.5
RYPSY	15.8	15.3	VYPFPGPIPNSLPQ	21.3	20.8	42.1	85.4
FRQF	18.4	18.0	TDVENLHLPLPL	22.7	22.5	40.6	56.2
FLPYPY	21.9	21.5	GPFPIIV	22.1	21.0	31.7	62.8
VAPFPEVF	22.4	23.7	EVLNENLLR	18.2	20.8	29.7	17.1
HLPLPL	21.5	20.3	VPPFLQPEVM	21.3	21.8	38.8	58.1
VYPFPGPIPN	20.7	20.7	TPVVVPPFLQPEVM	22.4	24.0	51.4	74.2
AVRSPAQIL	18.8	19.1	HQGLPQEVLNENLLRFF	23.1	25.6	53.7	58.2
RDMPIQ	12.8	14.8	EGIHAQQREPMIG	15.8	14.6	23.4	20.4
IHPFAQ	22.0	21.5	SQSKVLPVPQK	14.1	15.1	17.5	20.3
AVRSPAQIL	19.9	19.1					
KYKVPQL	19.3	16.8					
KRNAVPITP	16.8	16.4					
KVLPVPQ	16.4	17.9					
RDMPIQ	14.8	14.8					
HKEMPFPK	15.4	16.7					
YPVEPFTESQ	17.6	17.7					
LTEEEKNRLNFLKK	17.6	16.9					
HQGLPQEVLNENLLR	20.0	20.4					
VAPFPEVFGK	20.5	20.2					
DMPIQAFL	22.4	21.4					
DMPIQAFLLY	23.7	24.4					
YPSYGLNYY	19.7	20.2					
EMPFPK	16.6	16.7					
ALPQYLK	17.1	18.4					
AMKPWIQPK	17.3	17.4					
DMPIQAF	20.1	20.0					
VAPFPEVFGK	20.6	20.2					
LYQGPIVLNPWDQVKR	20.9	20.7					
FVAPFPEVFGK	21.9	22.8					
SPAQILQWQVL	22.2	22.5					

Retention times of peptides were predicted on basis of the retention time coefficients of the constituent amino acids, using the coefficients given by [a]Guo et al. (1986a) and [b]Meek (1980).

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CHAPTER 3

Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics

ABSTRACT

Casein and whey protein were hydrolysed 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 relations between hydrolysate characteristics and emulsion properties. All whey protein hydrolysates formed emulsions with bimodal droplet size distributions. 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 droplet aggregates. 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.

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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 (Asselin et al., 1988). As an alternative, extensively hydrolysed proteins can be used in the diet, as nutritional value is preserved (Lakkis & Villota, 1990; Boza et al., 1994). Hydrolysed proteins might also be beneficial for patients suffering from specific digestion disorders like cystic fibrosis or short bowel syndrome (Farrell et al., 1987), or can be used in high protein diets in case of malnutrition (de Freitas et al., 1993; Frokjaer, 1994; Schmidl et al., 1994).

Enzymatic hydrolysis of proteins does not only affect digestibility and allergenicity of proteins, but also induces modification of functional properties like solubility, viscosity, gelation, and emulsifying and foaming properties (Chobert et al., 1988a; Mahmoud et al., 1992). Hydrolysis of proteins causes changes such as an increase of the number of charged groups, a decrease of the average molecular weight and exposure of reactive groups, factors influencing emulsion-forming and emulsion-stabilising abilities of protein hydrolysates (Nielsen, 1997; Caessens et al., 1999a).

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 (Walstra & Smulders, 1997). During the formation of an oil in water emulsion in a homogeniser, surfactant molecules adsorb on the interface of droplets, droplets break up into smaller droplets and newly formed droplets collide, possibly resulting in coalescence. The final droplet size is an equilibrium between droplet break up and droplet coalescence. Surfactants (*e.g.* protein or peptides) contribute to the formation of droplets by lowering the interfacial tension (facilitating droplet break up) and by prevention of coalescence (Walstra, 1993). Once emulsion droplets are formed, they are subject to several forms of instability. The three main factors of instability are creaming, primarily depending on droplet size, aggregation and coalescence. Emulsion stability is favoured by proteins and peptides that are able to oppose attraction between emulsion droplets by promoting electrostatic repulsion or steric hindrance (Damodaran, 1997; Smulders et al., 1999).

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 (Chobert et al., 1988a; Haque & Mozaffar, 1992) and low DH whey protein hydrolysates (Haque & Mozaffar, 1992; Vojdani & Whitaker, 1994) in comparison to the parental materials. On the other hand, reduction of emulsion-forming ability after hydrolysis was also reported for casein hydrolysates emulsions (Chobert et al., 1988b; Slattery & Fitzgerald, 1998) and emulsions of whey hydrolysates (Turgeon et al., 1992). The emulsion stability generally decreases with hydrolysis for all milk proteins, also at low DH values (Chobert et al., 1988a; Haque & Mozaffar, 1992; Agboola & Dalgleish, 1996). 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 (Mutilangi et al., 1996). Chobert and coworkers (Chobert et al., 1988a) 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 (Singh & Dalgleish, 1998) reported that a peptide length of only 500 Da is needed for emulsion stabilisation. It is commonly thought that peptide size is not the only factor influencing emulsion behaviour of peptides. As was shown by several authors, amphiphilicity of peptides is important for interfacial and emulsifying properties of peptides (Gauthier et al., 1993; Caessens et al., 1999b; Rahali et al., 2000). Rahali and coworkers (2000) analysed 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, 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 characterised 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 standardised manner and to characterise all hydrolysis products with the same methods. Additionally, the data obtained from this approach can be statistically analysed, 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 hydrolysed with eleven different commercially available enzymes to various degrees of hydrolysis, in order to study hydrolysates that are similar to commercially available products. All hydrolysates were characterised according to standard protocols. The results were analysed 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) 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 obtained from Merck or Biosolve. Milli-Q water was prepared with a Millipore system, 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) and UV detector (SPD-10Avp) or fluorescence detector (RF-10Axl).

Production of hydrolysates

Casein and whey protein concentrate were hydrolysed with eleven 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 (Newlase F), Röhm (Corolase PP, Corolase L10) and Valley Research (Validase FP). Protein suspensions or solutions of 800 ml 5% (w/w) protein were hydrolysed in a pH-Stat set-up (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 hours of hydrolysis) and sample time in final hydrolysis were based on these preliminary results. Samples (200 ml) were taken at 1/3, 2/3 and the maximum degree of hydrolysis. Enzymes were inactivated by heating for 15 min at 90 °C. The hydrolysate was centrifuged (30 min, 3000x*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, DH=6%. Protein and enzyme codes are given in Table 1.

Table 1: Hydrolysis conditions.

Enzyme	pH	Temp (°C)	E/S[a] (%)	
			Casein (Cn[b])	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] 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.

Protein determination

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

Degree of hydrolysis

Degree of hydrolysis was measured spectrophotometrically according to the method of Adler-Nissen (1979) adapted for use in microtiter 96-wells plates. 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 standard curve. Sample solution (15 µl) was mixed with 45 µl of 0.21 M sodium phosphate buffer, pH 8.2, and 45 µl 0.05% of TNBS in a well. The covered well plate was incubated for 1 hour 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 % relative to the total area under the chromatogram.

Free amino acid content

Solutions of hydrolysate supernatants (0.02 à 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 a paper filter (Schleicher & Schluell, 595 ½). Non-clear 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 analysed by precolumn derivatisation with OPA reagent (similar to Burbach et al. (1982)), followed by separation on a reversed phase C18 column (Superspher 100 RP-18(e), 125x4 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 and 2% 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 & 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 % (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 the 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. (1997). A total of two ml tricaprylin oil (Sigma) was added to 18 ml hydrolysate solution and was mixed by hand-shaking during 40 seconds to obtain a pre-emulsion. The pre-emulsion was subsequently homogenised in a laboratory-scale high-pressure homogeniser (Delta Instruments, Drachten, the Netherlands) for 9 passages at 60 bar.

Emulsion-forming ability was investigated by measuring particle size distribution directly after homogenising 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 in the multivariate data-analysis section.

The emulsion stability was determined according to the method described by Pearce and Kinsella (1978), by measuring the turbidity at 500 nm of samples diluted 100 times in 0.1% SDS at t=0,1,3 and 24 hours. Samples, diluted 10 times, were checked for remaining aggregates using 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 hours. 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 (The 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, normalised (mean normalisation) particle size distribution curves were used as *x*-variables and emulsion stability after 24 hours 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 ~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 <500 Da. Free amino acid content ranged between 0.04 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]	< 0.1 – 12	0.1 – 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 (~pH 5) it precipitates. Hydrolysis in this pH-range resulted in partial resolubilisation 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 demineralised 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

To examine the emulsion-forming abilities of the protein hydrolysates, the emulsion droplets were studied by measuring the particle size distribution immediately after homogenisation. Particle size distributions can be summarised using various average particle diameters, *e.g.* 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 versus CnPx618) differ in the amount of large droplets.

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 hours relative to initial turbidity.

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 to 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 droplets. 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 ±0.33 μm and a main peak at large emulsion droplet sizes with d_{32} varying between 26 and 30 μm. The

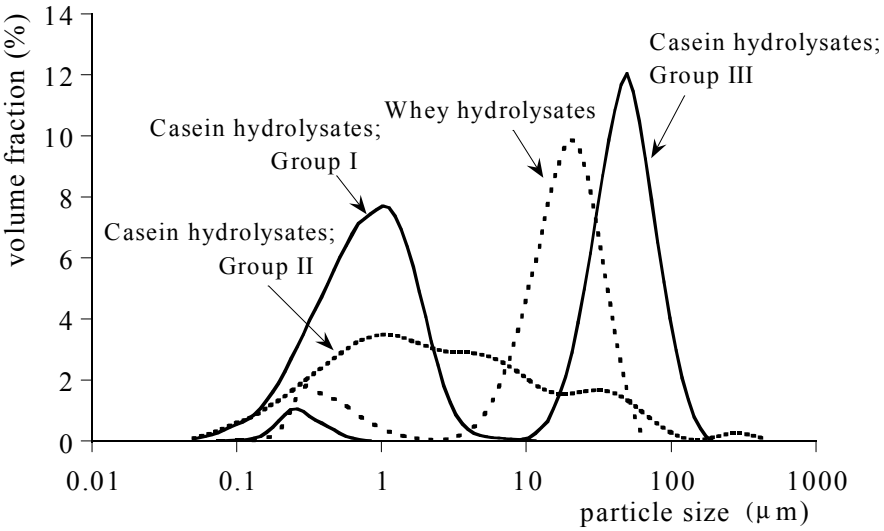

Figure 1: Particle size distributions of hydrolysate emulsions representing three types of casein emulsions and a typical whey hydrolysate emulsion. Distribution is expressed as volume % of oil included in droplets of each particle size. Samples used to represent groups were; group I: CnCl102, group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

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.

It should be noticed that the emulsions were diluted in water before estimation of their particle size distributions. Therefore, droplet aggregates might be responsible for the presence of the large particles. Indeed, microscopic examination of the emulsions after dilution in SDS showed that most emulsions belonging to group III mainly contained small emulsion droplets. Hydrolysates forming emulsions of group II contained both small and large emulsion droplets, indicating that coalescence had occurred to some extent. In conclusion, all hydrolysates were able to from small particles, but the emulsions differ in their ability to oppose immediate instability.

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 from 3.4 to 19 µm. The d_{43} values of the bimodal emulsions varied from 3.3 to 20.3 µm (Table 3). Microscopic examination of the emulsions after dilution in SDS showed that three hydrolysates (WcAlc23, WcNfw14, WcPx617) contained only rather large emulsion droplets, indicating that emulsion-forming ability of these hydrolysates was poor. Other emulsions contained either only small emulsion droplets or mixtures of droplets of different sizes.

In the present study none of the whey hydrolysates showed improved emulsion-forming ability compared to intact whey, while Lakkis and Vilotta (1990) measured a decrease in particle sizes and a more uniform distribution of emulsion droplets with whey protein hydrolysates prepared with pepsin, trypsin or chymotrypsin. Hydrolysis of β-lactoglobulin

(Caessens et al., 1999c) 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.* presence of large emulsion droplets after homogenising. Aggregation and creaming might promote coalescence, due to the increased contact time between oil droplets. Since coalescence is the final stage of emulsion 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 droplet aggregates, 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 (Voutsinas et al., 1983; Chobert et al., 1988a; Jackman & Yada, 1988; Huang et al., 1996; Lieske & Konrad, 1996; Slattery & Fitzgerald, 1998). 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 hours are given in Table 3. The major decrease in turbidity was observed during the first 1 to 3 hours (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 (2000). 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 to 5% of the total protein. After hydrolysis the enzymes are denatured and probably discarded with centrifugation. Hydrolysates from one enzyme differ in emulsion properties, indicating that if enzymes are not completely removed, they do not significantly influence emulsion properties.

Correlation between emulsion-forming 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 homogenisation. To investigate whether these particle size distributions are related to emulsion instability due to coalescence, regression analysis was performed with the size distribution as *x*-variables and

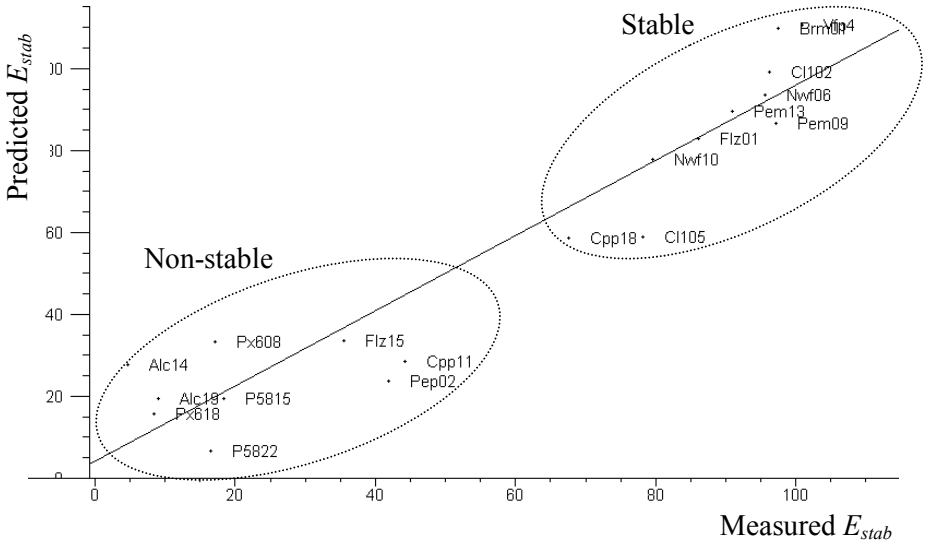

Figure 2: Correlation between predicted and measured emulsion stability (E_{stab}), expressed as remaining turbidity, of casein hydrolysate emulsions. Emulsion stability was predicted using particle size distribution curves of freshly made casein emulsion. Correlation coefficient (r) between predicted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

the stability 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 and a non-stable 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 III and are characterised by a narrow particle size distribution. Emulsions from hydrolysates of group I have a typical low average particle size (d_{32} of 0.56–0.95 μm) whereas stable emulsions of group III have a rather large average particle size (d_{32} of 2–5 μm). Although the emulsions of group III are stable against coalescence they are not stable against the formation of droplet aggregates and, therefore, cream immediately after homogenisation. The hydrolysates forming non-stable emulsions have a very broad particle size distribution (hydrolysates from group II) or the emulsions are composed of very large droplets with d_{32} >7 μm.

The broad particle size distributions of emulsions prepared with hydrolysates from group II indicate that these hydrolysates contain peptides that are surface active, enabling formation of small droplets, but that these hydrolysates are not able to stabilise the emulsion droplets.

Concerning whey protein hydrolysates, stability of emulsions as calculated from turbidity data was also correlated to the droplet size distributions of the emulsions. The correlation coefficient between measured and predicted values was 0.84 (data not shown). Generally, whey hydrolysate emulsions showed turbidity decrease 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 molecular weight distribution. 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 set-up, standard protein source and concentration), and as analytical methods were standardised, 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, and were therefore, studied in more detail.

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 hours.

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 values varying from 5.5 to 24%, all emulsions had similar droplet size distributions, confirming that the DH in that range does not correlate to emulsion-forming abilities. Also when the microscopic examinations were taken into account, no relation between the type of emulsions and DH values was observed.

According to the correlation coefficients for casein hydrolysate emulsions, no linear relation exists between d_{32} values and DH if all samples are analysed 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.

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.

Hydrolysates forming narrow emulsion droplet size distributions with low average particle sizes (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.

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 behaviour. A DH optimum for emulsion properties (Nielsen, 1997) or a linear relation between DH and emulsion-forming ability (Mahmoud et al., 1992) may therefore only be found if one protein/enzyme combination is considered.

Concerning the stability of emulsions, casein hydrolysates forming non-stable 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 hydrolysed to DH values >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 (Chobert et al., 1988a; Haque & Mozaffar, 1992; Caessens et al., 1999c).

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 the 3-4- and 2-3-kDa fractions for casein and the 2-3-kDa fraction for whey hydrolysates, were significant. A high proportion of high molecular weight peptides (>3 to 4 kDa) is positively related to emulsion stability, while a high proportion of peptides <2 kDa (low amount of high molecular peptides) results in non-stable 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 molecular weight distribution data could be reduced to five 'variables' describing the variance in 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 the 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 >2 kDa (peptide chain length ≅ 17 amino acids). Exceptions on this rule were observed, however. Two samples containing less than 65% peptides >2 kDa were stable. The prediction of the emulsion stability based on the MWD of these samples was good, which might point to 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 >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.

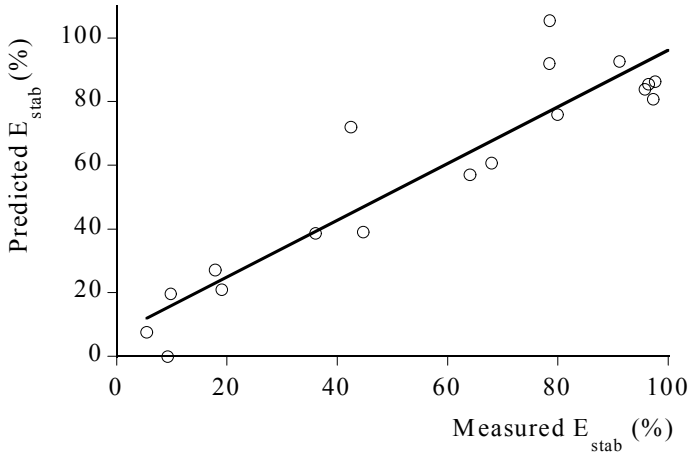

Figure 3: Correlation between predicted and measured emulsion stability (E_{stab}), expressed as remaining turbidity, of casein hydrolysate emulsions. Prediction was based on apparent molecular weight distribution of the hydrolysates. Correlation coefficient (r) between predicted and measured values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

Generally, high molecular weight peptides are mentioned to be beneficial for emulsion stability; the minimum size was estimated to be larger than 2 kDa (Lee et al., 1987; Caessens et al., 1999a), between 2.5 and 5 kDa (Agboola et al., 1998) or larger than 5 kDa (Chobert et al., 1988a). 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 with results found by Singh and Dalgleish (1998). 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 stabilise the emulsions.
In the present study, a correlation was found between emulsion stability and MWD of hydrolysates. In a recent study, Rahali and coworkers (2000) 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 et al. (2000).

In conclusion, it was shown that the emulsion-forming behaviour of hydrolysates is generally independent of molecular weight distribution and degree of hydrolysis. Emulsion instability was caused by creaming and coalescence. Creaming was observed in emulsions containing relatively large emulsion droplets, often already present directly after homogenisation. Coalescence of emulsion droplets was correlated to the MWD of the hydrolysates stabilising the emulsions. Hydrolysates with a high proportion of peptides with molecular weight >2 kDa formed emulsions that are relatively stable towards coalescence.

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CHAPTER 4

Correlations between biochemical characteristics and foam-forming and -stabilising ability of whey protein and casein hydrolysates

ABSTRACT

Whey protein and casein were hydrolysed with eleven 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 molecular weight distribution of whey hydrolysates as predictors showed that the hydrolysate fraction containing peptides of 3 to 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.

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INTRODUCTION

Proteins are used in all kinds of food products to profit from their nutritional value or from specific functional properties. Their physicochemical behaviour 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, and emulsion and foam properties. Choice of enzyme and process conditions influence hydrolysate composition and thereby the functional properties. This chapter 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 (Walstra & Smulders, 1997). 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 (Damodaran, 1997). 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 stabilise 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 towards the area with high surface tension, resulting in a liquid flow to the thin part of the film (Damodaran, 1997).

Molecular mechanisms influencing foam formation and foam stability in protein foams have been studied for some isolated proteins (for a review see Damadoran (1997)). 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 (Wilde & Clark, 1996). On the other hand, peptides formed during hydrolysis might destabilise protein foams by displacement of proteins or by disturbing protein/protein interactions (Zhu & Damodaran, 1994; Wilde & Clark, 1996). 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 (Lorient et al., 1989; Zhu & Damodaran, 1994; Ibanoglu & Ibanoglu, 1999).

Hydrolysates contain a variety of peptides, differing in their functional behaviour. Fractions obtained from a plasmin hydrolysate of β-casein showed clear differences in their foam-stabilising properties and in their interfacial behaviours; the surface pressure increase was higher for hydrophobic fractions than for amphipathic fractions (Caessens et al., 1997). 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 fractions showed that the presence of distinct hydrophilic and hydrophobic areas contributes to good interfacial properties (Turgeon et al., 1992).

Althouse and coworkers (1995) tested the foam-forming ability and foam stability of whey protein hydrolysates produced by five different enzymes. They showed that foam capacities (measured as % overrun) of hydrolysate permeates, obtained after ultrafiltration over a 10 kDa membrane, were higher than those of the corresponding retentates. Lieske and Konrad (1996) 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 (Abert & Kneifel, 1993; Ludwig et al., 1995) do not consider relationships with 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 emulsion properties of casein hydrolysates, which are also of interest as formation and stabilisation of foams and emulsions are governed by essentially similar interfacial properties (Damodaran, 1997; Walstra & Smulders, 1997). For casein hydrolysates both increased and decreased emulsion-forming abilities were measured with hydrolysates prepared with various enzymes, having different degrees of hydrolysis (Chobert et al., 1988a; Chobert et al., 1988b; Haque & Mozaffar, 1992; Agboola & Dalgleish, 1996). Emulsion stability generally decreased upon hydrolysis (Chobert et al., 1988a; Chobert et al., 1988b; Haque & Mozaffar, 1992). Emulsion stability towards coalescence was shown to be correlated to a high proportion of peptides larger than 2 kDa (Chapter 3).

Comparison of foam characteristics of protein hydrolysates reported in literature is difficult because foam characteristics strongly depend on the methods used to prepare foams and methods used to analyse foam formation and stabilisation (Phillips et al., 1990; Mutilangi et al., 1996; Wilde & Clark, 1996). Moreover, foam properties depend on solvent conditions such as pH (Caessens et al., 1997) and ionic strength (Zhu & Damodaran, 1994), 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 hydrolysed 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 analysed 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), whey protein (WPC 60) from Milei GmbH (Stuttgart, Germany). Acetonitrile, tetrahydrofuran, methanol and tris were purchased from Biosolve, 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 Millipore system, 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 hydrolysed with eleven different enzymes, under conditions as given in Table 1, based on optimum hydrolysis conditions as given by the manufacturers.

Table 1: Hydrolysis conditions.

Enzyme	pH	Temp (°C)	E/S[a] (%)	
			Casein (Cn[b])	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] 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.

Enzymes were obtained from Novo Nordisk (Pem, Flavourzyme, Alcalase), Biocatalyst (Promod 184, Promod 258, Pepsin), Genencor (Protex 6L), Amano (NewlaseF), Röhm (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 hydrolysed in a pH-Stat set up (Titrino 718, Metrohm). Whey protein suspensions were pre-treated for 15 min at 90 °C to improve accessibility of the proteins to the proteolytic enzymes (Reddy et al., 1988); casein solutions were not pre-treated. 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 hours of hydrolysis) and sample time in final hydrolysis were based on these preliminary results. Samples (200 ml) were taken at 1/3, 2/3 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., 3000x*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.
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 2 digits for protein source, 3 digits representing the enzyme used and 2 digits encoding the degree of hydrolysis reached, for example, CnNwf06 means 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-analyser (NA 2100 Protein, CE instruments). For calculation of protein concentrations a Kjeldahl factor of 6.38 was used.

Degree of hydrolysis
Degree of hydrolysis was measured spectrophotometrically according to the method of Adler-Nissen (1979), adapted for use in microtiter 96-wells plates. Hydrolysate samples (total hydrolysate) were diluted in 1% SDS solution to a concentration of 0.05% (w/v on protein basis); 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 hour 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 MWD of supernatants was determined by size exclusion chromatography (SEC) using a TSK-gel G 2000 SWXL (7.8x300 mm column, Toso Haas) connected to a TSK-gel SW pre-column (7.5x75 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

kDa, 15-20 kDa, 10-15 kDa, 7-10 kDa, 5-7 kDa, 3-5 kDa, 1-3 kDa, <1 kDa. The proportion of each fraction was expressed as % relative to the total area under the chromatogram.

Free amino acid content

Solutions of hydrolysate supernatants (0.02 à 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 a paper filter (Schleicher & Schluell, 595 ½). Non-clear 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 analysed by pre-column derivatisation with OPA reagent (similar to method of Burbach et al. (1982)), followed by separation on a reversed phase C18 column (Superspher 100 RP-18(e), 125x4 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. Total amino acid content of the samples was calculated by summing individual amino acid concentrations. Free amino acid content was expressed as % (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 protein) were dissolved in 1 ml 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 Volt hours). 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 weighted and protein content was determined. Solubility was expressed as proportion (%) of protein in supernatant, relative to total protein content of the 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. (1997). 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 hour. For statistical analysis foam stability was expressed as the % foam volume remaining after 15 min (Vf_{15}) or after one hour (Vf_{60}) relative to the initial foam volume.

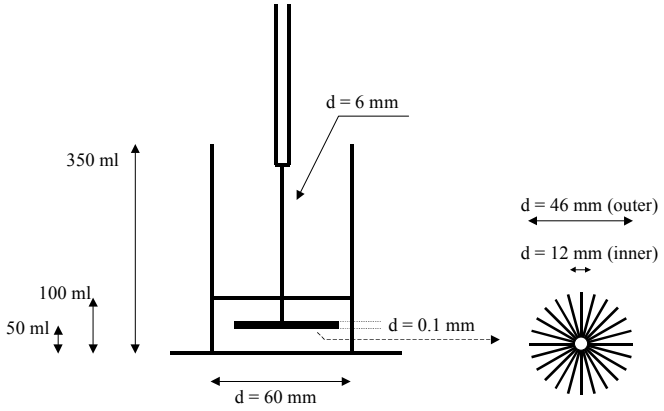

Figure 1: Schematic representation of the experimental set-up for the foam formation and stability test with a detail of the small impeller used, d = diameter. From Caessens et al (1997), with permission.

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 standardised. As the molecular weight fractions are mutually correlated, normal 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, representing 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

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 models was determined by calculation of the correlation between the predicted and measured values for the studied functional property.
Regression analysis was also performed with complete SEC 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.

Table 2: Properties of casein and whey hydrolysates used for foam experiments. Values represent the highest and the lowest values measured over all casein or whey samples included in correlation studies (for details see appendix).

	Casein	Whey
DH (%)	0.5 – 22	5.5 – 24
Free amino acids (%)[a]	<0.1 – 12	0.1 – 23
Solubility (% protein)[b]	38 – 85	18 – 96
Apparent MWD (%)[c]		
>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] 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.

Whey protein and casein were (partly) hydrolysed 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 a 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 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 Chapter 3. Differences between the two columns were expected because electrostatic and hydrophobic interactions interfere with the

separation on hydrodynamic volume (Visser et al., 1992; Fujinari & Manes, 1997; Tossavainen et al., 1997). These interactions depend on the column material and the eluents used in SEC (Barth, 1982; Smyth & Fitzgerald, 1997). 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 non-foaming 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 hypothesised that the peptide concentration (0.05%) used for foaming experiments in our study was too low to increase foam-forming ability. Althouse and coworkers (1995) studied whey hydrolysate foams prepared with 5% hydrolysate at pH 7 and found improved foam capacity (% overrun). Foam-forming abilities of whey protein concentrates depend on the protein concentration, with a reported optimum of ~10% (Schmidt et al., 1984). However, 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) (Van Koningsveld, 2001). 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 (Caessens et al., 1999). In conclusion, the protein concentration should be sufficiently high to measure improved foam-forming ability.
The different results obtained with the β-lactoglobulin hydrolysates (Caessens et al., 1999) and the whey protein hydrolysates of the present study may be attributed to differences in degree of hydrolysis, which are 1-4% and >5.5% respectively, to the use of an isolated protein instead of a whey 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 foams, but the foams were highly unstable, resulting in almost complete disappearance of the foams within the first minute after whipping.
The good foam-forming ability of casein is also measured by others (Grufferty & Mulvihill, 1991; Lee et al., 1992) and can be attributed to the high flexibility of the protein. A casein hydrolysate with a high degree of hydrolysis prepared with Alcalase showed significant decrease in foam capacity (% overrun) compared to intact protein (Townsend & Nakai, 1983), 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 (Ludwig et al., 1995). 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 (Abert & Kneifel, 1993). Foam-forming ability and foam 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% (Slattery & Fitzgerald, 1998).
It was shown that all tested casein hydrolysates are able to form high initial foam levels, whereas foam-forming abilities of whey hydrolysates differ. Foam formation is governed by three factors: transportation, penetration and reorganisation of the molecules on the air/water

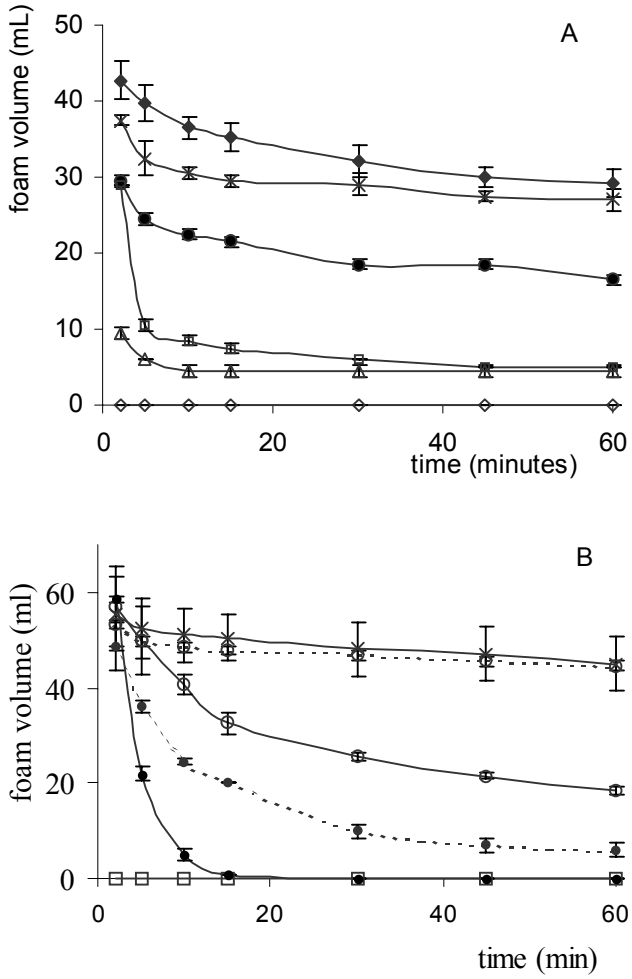

Figure 2: Foam volumes as produced with various whey protein hydrolysate (A) and casein hydrolysate (B) solutions at pH 6.7, as a function of time after whipping (means of duplicate measurements). A) (♦) WPC60, (×) WcCpp09, (•) WcPem06, (□) WcPx611, (△) WcP5807, (◇) WcVfp06. B) (--○--) Casein, (□) CnAlc19,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

interface. These processes depend on size, surface hydrophobicity and structural flexibility of the surfactants (Wilde & Clark, 1996). The variation in DH and MWD between the whey- and the casein hydrolysates is comparable (Table 2), so differences in MWD or DH cannot explain the contrasting behaviour. For adsorption on the air/water interface molecules should contain hydrophobic regions (Horiuchi et al., 1978; Turgeon et al., 1992). 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 (Schmidt et al., 1984). 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, *i.e.* 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 stabilise foams (Table 3). The first group of whey protein hydrolysates forming stable foams (WI) consists only of WcCpp09. The second group of whey protein 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,

Table 3: Grouping of whey and casein hydrolysates according to their foam stabilities, and for group WIII according their foam-forming abilities.

	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 remaining foam after 1 hour, 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] For abbreviations see Table 1.

showing curves represented by sample WcPem06 in Figure 2A. The whey protein 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 case of whey hydrolysates.

In literature both increased and decreased foam stabilities of whey hydrolysate foams are reported (Lakkis & Villota, 1990; Perea et al., 1993; Lieske & Konrad, 1996).

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 casein hydrolysates was able to stabilize the hydrolysates very well; the foam stability was comparable to that of intact casein foam. Three hydrolysates (CnVfp04, CnVfp07 and CnNwf06) belong to this group. The second group consists of casein hydrolysates that stabilised the foams to some extent; the remaining of foam volume over 1 hour varied from 10 to 70%. The third group includes non-stable foams; all foam disappeared within 1 hour. 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 case of 7 of the 11 enzymes used. This might point to the fact that the enzyme specificity influences the stability of casein hydrolysate stabilised foams, in contradiction to the absence of enzyme influence observed with the whey hydrolysates.

In many casein foams destabilisation 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 neighbouring bubbles or two bubbles melted together, resulting in larger bubbles that finally broke. In case of whey hydrolysates this was also observed, but in fewer samples than with casein hydrolysates.

Destabilisation 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 destabilisation mechanisms in protein hydrolysate stabilised foams. Caessens and coworkers (1999) investigated stability of 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, since the study was aimed at comparing final hydrolysate products. Enzymes constitute 0.5 to 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-stabilising 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, since their foam had already collapsed before the first measuring point.

Table 4: Correlation coefficients between biochemical properties and foaming properties of whey protein and casein hydrolysates.

	Foam properties					
	Whey protein hydrolysates			**Casein hydrolysates**		
Biochemical properties	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] Initial foam volume.
[b] % Foam volume remaining after 15 min (in % relative to foam volume at t=0).
[c] % Foam volume remaining after 60 min (in % relative to foam volume at t=0).

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 non-stable foams (group CIII) had DH values >14%. From the ten 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.

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 foam levels. Therefore, the foam-forming ability does not seem to depend on the molecular weight distribution of the peptides. Whey hydrolysates, however, did show differences in their foam-forming ability. Calculation of the correlation coefficients between the MW 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 with peptides >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, while for regression analysis all molecular weight fractions, which are interdependent, are used. As the proportions 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 ability almost as accurately as does the complete MWD. Study of the proportion of this fraction in the hydrolysates showed that, if the fraction 3-5 kDa represented more than 10% of all peptides, generally more than 25 ml foam was formed (data not shown). Remarkably, the MW fraction >20 kDa does not contribute to the foam-forming ability of hydrolysates, while intact whey is able to form a voluminous foam. The MW fraction >20 kDa contains remaining intact protein and/or peptide aggregates, which cannot be distinguished from each other with the chromatographic

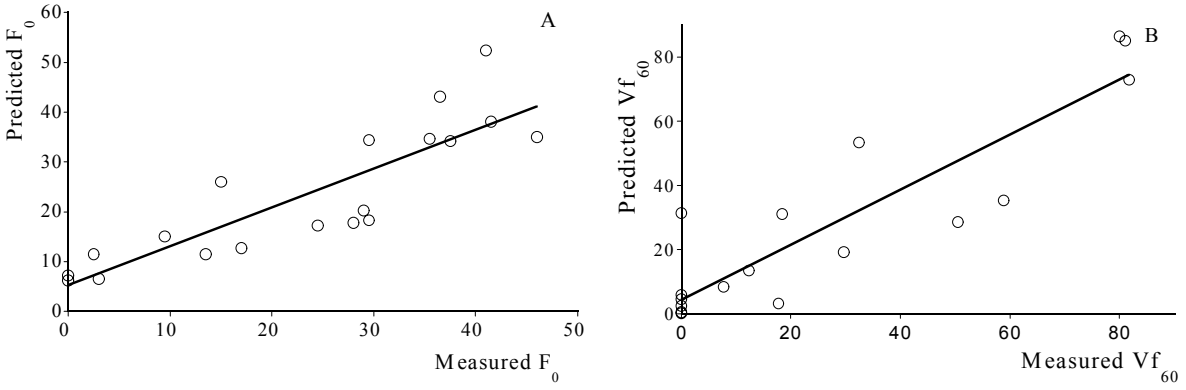

Figure 3: Correlation between predicted and measured values for (A) initial foam volume (F_0) of whey hydrolysate foams (r = 0.86) and (B) for remaining foam volume (Vf_{60}) values of casein hydrolysates (r = 0.90). Prediction was based on multivariate regression analysis (PLS) with molecular weight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

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 since 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 solutions used to test foam properties, their influence on foam-forming ability was probably of minor importance, since especially differences in the lower molecular weight fractions are responsible for the differences in foam-forming abilities.
Althouse et al. (1995) 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 foam-forming abilities of whey and casein hydrolysates it might be hypothesised 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 that amphiphilic peptides are present 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 molecular weight distribution 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 favoured over larger peptides might be explained by the faster diffusion of low-MW peptides, and possibly by 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 their MWD, as shown by the rather high correlation coefficients (Table 4). Fractions with positive correlation coefficients contribute to stabilisation of the foam, since 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.
The regression coefficients (data not shown) had positive values for the fractions with MW >7 kDa. The hydrolysates forming the most stable foams contained more than 25% peptides >10 kDa. To investigate whether the foam stability of these samples arises from the presence of

intact casein, the samples were analysed with SDS gel electrophoresis to study the composition of the high molecular weight fraction (Figure 4). The analysis showed that sample CnNwf06 contained a relative 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, while 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 stabilisation of the foam.

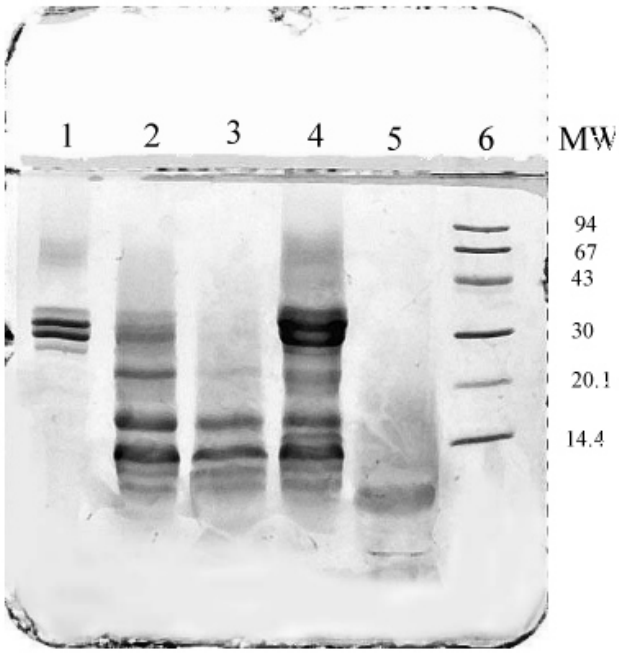

Figure 4: Determination of the presence of intact casein in casein hydrolysates using SDS-PAGE. Lane 1 sodium caseinate, lane 2-5 casein hydrolysates: CnVfp04 (2), CnVfp07 (3), CnNwf06 (4), CnCpp11 (5), lane 6 molecular weight (MW) marker.

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 behaviour of whey protein (Morr & Ha, 1993; Zhu & Damodaran, 1994). The mineral concentration in hydrolysates is not expected to influence results of foam experiments in the present study, since 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 WPC 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 non-protein material present and foam properties was not

Appendix: Properties of casein and whey protein hydrolysates used in foam experiments.

Sample	Molecular weigth fractions (kDa)								faa[a]	Sol[b]	Foam properties	
Casein	<1	1-3	3-5	5-7	7-10	10-15	15-20	>20	(%)	(%)	F_0[c]	Vf_{60}[d]
CnAlc14	34	48	13	2.7	1.4	0.6	0.2	0.4	0.7	79	8.5	0
CnAlc19	48	46	4.5	0.5	0.2	0.0	0.0	0.5	2.4	78	0	0
CnBrm01	13	34	19	11	9.4	6.8	2.7	4.2	0.7	47	51	59
CnBrm06	18	45	19	8.7	5.1	2.7	0.8	0.9	2.8	59	52	8
CnC1002	13	32	17	11	9.9	7.4	3.6	5.9	0.9	58	57	32
CnC1005	17	37	18	9.8	7.9	5.3	2.2	2.9	0.9	61	63	18
CnCpp11	21	32	20	9.1	6.5	5.7	2.3	2.8	40	76	53	50
CnCpp18	35	46	13	3.1	1.8	1.0	0.4	0.7	72	83	52	43
CnFlz15	33	49	10	2.5	1.6	1.2	1.0	0.9	84	54	33	0
CnNwf06	12	23	17	11	9.0	9.3	6.5	12	1.1	48	58	80
CnNwf10	23	41	19	7.1	4.3	2.8	1.4	1.9	2.4	58	59	54
CnP5815	43	49	5.8	1.1	0.5	0.3	0.1	0.2	13	77	43	0
CnP5822	51	44	3.7	0.6	0.3	0.2	0.1	0.1	29	85	44	0
CnPem09	22	41	17	6.7	5.4	3.8	1.8	2.2	0.4	80	54	30
CnPem13	33	49	12	2.6	1.1	1.3	0.9	1.1	0.7	80	62	18
CnPep02	18	21	13	5.7	8.6	7.2	4.2	22	1.5	42	64	47
CnPep06	28	29	18	9.9	6.8	4.7	1.1	1.9	2.9	51	59	0
CnPx608	23	40	19	8.2	5.6	2.8	1.0	1.4	1.8	65	49	12
CnPx618	45	47	6.5	0.9	0.3	0.1	0.0	0.2	4.9	85	3	0
CnVfp04	21	23	14	7.6	8.3	7.6	3.9	15	3.2	59	52	82
CnVfp07	21	22	13	7.7	6.8	6.5	2.9	20	9.4	57	56	81

Whey	<1	1-3	3-5	5-7	7-10	10-15	15-20	>20	faa[a]	Sol[b]	F_0[c]	Vf_{60}[d]
WcAlc06	29	40	14	4.1	3.6	3.2	1.8	4.6	0.9	66	46	7
WcAlc23	60	36	2.6	0.6	0.5	0.4	0.1	0.2	5.3	96	0	0
WcBrm05	13	35	17.4	8.5	6.1	4.6	2.3	14	2.1	56	41	0
WcBrm06	14	37	17	7.6	5.0	3.8	2.0	14	2.7	64	37	49
WcCl106[d]	14	31	14	6.6	5.9	5.5	3.0	21	1.6	55	13	0
WcCl107	11	27	13	6.9	6.4	6.2	3.5	25	1.9	56	36	0
WcCpp09	17	32	12	6.9	6.0	5.4	3.3	17	54	72	38	72
WcCpp12	31	45	9.1	3.8	3.0	2.7	1.3	4.1	97	88	28	61
WcFlz09	27	44	9.1	3.9	2.7	2.3	1.2	9.9	99	47	14	0
WcFlz24	41	42	6.0	2.5	2.1	1.5	0.9	3.1	227	57	2.5	0
WcNwf07	22	47	11	3.2	3.4	3.5	1.9	8.5	3.8	28	25	0
WcNwf14	40	47	5.2	1.5	2.1	2.0	0.6	2.2	7.5	39	3	0
WcP5807	38	47	9.5	2.0	1.2	0.7	0.3	1.1	8.8	53	9.5	0
WcP5815	43	41	7.8	1.7	1.4	1.2	0.6	3.2	26	56	17	0
WcPem06	31	39	11	3.6	3.7	3.9	2.8	5.1	1.8	60	30	56
WcPem10	47	38	7.6	2.7	2.1	1.4	0.7	1.1	5.0	67	30	63
WcPep05	12	26	13	8.3	5.8	6.6	4.1	24	2.1	35	42	40
WcPep10	25	43	12	4.7	3.1	2.7	1.4	7.7	3.2	40	15	0
WcPx611	38	42	9.4	3.0	2.3	2.0	0.9	2.5	2.1	66	29	0
WcPx617	61	35	2.5	0.8	0.5	0.3	0.1	0.2	6.2	94	0	0
WcVfp06[d]	10	16	9.1	5.8	7.3	13	10	28	3.0	31	0	0
WcVfp07[d]	8.9	14	7.8	5.3	7.9	15	13	27	5.1	18	15	0

[a] Free amino acid content.
[b] Initial foam volume.
[c] % Foam volume remaining after 60 min relative to initial foam volume.
[d] Samples were excluded from calculation of correlations between MWD and foam properties.

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.

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CHAPTER 5

FTIR spectra of whey and casein hydrolysates in relation to their functional properties

ABSTRACT

Mid-Infrared spectra of whey and casein hydrolysates were recorded using Fourier Transform Infrared (FTIR) spectroscopy. Multivariate data analysis techniques were used to investigate the capacity of FTIR spectra to classify hydrolysates and to study the ability of the spectra to predict bitterness, solubility, emulsifying and foaming properties of hydrolysates. Principal component analysis revealed that hydrolysates prepared from different protein sources or with different classes of proteolytic enzymes are distinguished effectively on basis of their FTIR spectra. Moreover, multivariate regression analysis showed satisfactory to good prediction of functional parameters, the coefficient of determination (R^2) varied from 0.60 to 0.92. The accurate prediction of bitterness and emulsion-forming ability of hydrolysates by using only one uncomplicated and rapid analytical method has not been reported before. FTIR spectra in combination with multivariate data analysis proved to be valuable in protein hydrolysate fingerprinting and can be used as an alternative for laborious functionality measurements.

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INTRODUCTION

Proteins are nutritionally important as a source of nitrogen and essential amino acids. For several purposes it is beneficial to hydrolyse proteins, for example to reduce allergenicity (Asselin et al., 1988; Cordle, 1994), to achieve specific dietary requirements (Farrell et al., 1987; de Freitas et al., 1993; Frokjaer, 1994; Clemente, 2000) or to improve functional properties (Adler-Nissen, 1986a; Nielsen, 1997). Hydrolysis of proteins leads to numerous alterations in protein functional characteristics, like changes in solubility, viscosity, taste, emulsion- and foam-forming ability and emulsion- and foam stability (Nielsen, 1997). These functional parameters are important for application of hydrolysates in food products. Their determination is laborious, hence, substitution of functionality measurements by easy, fast analytical methods is of great interest, both for product development and for development of fast and accurate quality control applications. Therefore, research concerning correlations between analytical parameters and functional properties is desirable.

Protein hydrolysates are often characterised by their degree of hydrolysis (DH) and the molecular weight distribution (MWD) of the constituent peptides, the latter usually being determined using size exclusion chromatography. Concerning whey and casein hydrolysates, MWD is correlated to emulsion stability of both casein and whey hydrolysate emulsions (Chapter 3), to foam-forming properties of whey hydrolysates and to foam stability of casein hydrolysate foams (Chapter 4). Emulsion-forming properties, however, could not be related to MWD (Chapter 3).

Another important quality parameter for protein hydrolysates is their bitterness, which is known to originate from certain peptides formed during proteolysis (for reviews about bitter peptides see Adler-Nissen, 1986b; Adler-Nissen, 1987; Lemieux & Simard, 1992; Tanimoto et al., 1992). Determination of perceived bitterness is laborious, since sensory panels are needed. Reversed phase chromatography, that principally separates peptides based on their hydrophobicity, is often used to identify peptides that cause bitterness of hydrolysates (Minagawa et al., 1989; Lovsin Kukman et al., 1995; Lin et al., 1997). However, bitterness of hydrolysates cannot be predicted adequately from reversed phase chromatography or other analytical methods.

Next to chromatographic methods, spectroscopic methods like fluorescence or infrared (IR) spectroscopy are currently used to characterise food and food-related products. Infrared spectroscopy is based on the absorption of radiation due to vibrations between atoms in a molecule and, therefore, provides information about the chemical composition and conformational structure of food components (Willard et al., 1981). The fingerprint region of the IR spectrum, which is the region from 1800 to 800 cm^{-1}, is often a very useful part for analysis of proteinaceous material (Al Jowder et al., 1999), since in this range the bonds forming the amide group (C=O, N-H and C-N) absorb. The two most important vibrational modes of amides are the amide I vibration, caused primarily by the stretching of the C=O bonds, and the amide II vibration, caused by deformation of the N-H bonds and stretching of C-N bonds. The amide I vibration is measured in the range from 1700 to 1600 cm^{-1} and the amide II region from 1600 to 1500 cm^{-1} (Curley et al., 1998). The exact frequencies at which

these bonds absorb depend on the secondary structure of the proteins or peptides (Kumosinski & Farrell, 1993; Curley et al., 1998).
For protein research, infrared spectroscopy has been applied to study secondary structure of proteins (both in solid state and in solution) (Casal et al., 1988; Kumosinski & Farrell, 1993; Belton et al., 1997; Forato et al., 1998) and for qualitative or quantitative determination of proteins in mixtures (Bertrand et al., 1987; Rodriguez-Saona et al., 2000; Sasic & Ozaki, 2001). Because IR spectra include information regarding (secondary) structure of peptides combined with information about functional groups it is interesting to investigate its suitability for characterisation of protein hydrolysates.
As protein hydrolysates are mixtures of peptides it will be very difficult, if not impossible, to identify specific peptides. However, to describe hydrolysates and to study correlations between IR spectra and functional properties, it is not necessary to elucidate the exact composition of the hydrolysates; the hydrolysate can be considered as a 'black box' system, characterised by the IR spectrum. The relation between the spectra and the functional properties can be studied with multivariate statistical analysis, which has been proven to be a powerful tool in investigating such relationships (Josefson & Tekenbergs-Hjelte, 1991; Bietz & Simpson, 1992; Pribic, 1994; Seiden et al., 1996; Forina et al., 1998; Rodriguez-Saona et al., 2000).
In the present study, FTIR spectra of casein and whey hydrolysates were recorded and were correlated to bitterness, solubility and emulsion and foam properties, using multivariate regression analysis in order to investigate whether FTIR spectra can substitute laborious functional tests.

MATERIALS AND METHODS

Materials

Sodium caseinate (89% protein based on dry weight) was obtained from DMV International (Veghel, The Netherlands), whey protein (WPC 60) from Milei GmbH (Stuttgart, Germany).

Production of hydrolysates

Whey and casein hydrolysates were produced as described previously (Chapter 2). Protein suspensions or solutions (5% (w/w) protein) were hydrolysed in a pH-Stat set-up using eleven different enzymes, under conditions as given in Table 1. Samples were taken at 1/3, 2/3 and at the maximum degree of hydrolysis. Enzymes were inactivated by heating for 15 min at 90 °C. The hydrolysate was centrifuged (30 min., 3000x*g*, 20 °C) at the pH of hydrolysis. Supernatant and pellet were separated. Supernatant was freeze-dried and used for further studies. Sample codes are subsequently composed of 2 digits for protein source, 3 digits representing the enzyme used and 2 digits encoding the degree of hydrolysis reached, for example CnNwf06: casein, Newlase F, DH=6%. Protein and enzyme codes are given in Table 1. Enzymes were denoted 'acidic', 'neutral' or 'alkaline', according to the pH at which they are active, respectively acidic pH (pH 3), neutral pH (pH 5-7) and alkaline pH (pH 8). In total, 33 casein and 33 whey hydrolysates were produced.

Table 1: Hydrolysis conditions.

Enzyme	pH	Temp (°C)	E/S[a] (%)	
			Casein (Cn[b])	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] 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.

FTIR spectroscopy

The method of choice for IR analysis is the Fourier Transform Infrared (FTIR) technique, which is the most commonly used method for mid-infrared spectroscopy (Larrabee & Choi, 1993). Spectroscopic measurements were performed using approximately 25 mg freeze-dried hydrolysate supernatant mixed with 225 mg dried KBr (10% w/w). The FTIR spectra between 1800 and 800 cm^{-1} were recorded using a BioRad FTS-60A FTIR spectrometer using the DRIFT (diffuse reflectance) mode. The interferometer as well as the chamber that housed the detector were purged with dry nitrogen to remove spectral interference resulting from water vapour and carbon dioxide. All spectra were obtained at room temperature at a resolution of 8 cm^{-1} and 64 interferograms were co-added for a high signal-to-noise ratio. Triangular apodization was employed. The single beam spectrum of KBr was subtracted from the single beam spectrum of each protein hydrolysate to obtain the desired spectrum. All experiments were performed in duplicate. Prior to data analysis the spectra were baseline corrected (two points, 1801.5, 778.4 cm^{-1}) and normalised (mean normalisation option).

Protein determination

Protein concentration was measured by determination of total nitrogen on an N-analyser (NA 2100 Protein, CE instruments). For calculation of protein concentrations a Kjeldahl factor of 6.38 was used (Adler-Nissen, 1986a).

Solubility

Solubility was expressed as the amount of supernatant protein relative to the total protein content before hydrolysis (% w/w).

Emulsion forming and stability

Emulsion-forming ability and emulsion stability of 22 casein and 22 whey hydrolysates, randomly selected from the 33 hydrolysates from each protein source, were measured as previously described (Chapter 3). In summary, 18 ml of 0.56% (w/v) supernatant protein in 0.02 M imidazole/HCl buffer pH 6.7 with 3.44 g/l NaCl was emulsified with 2 ml of Tricaprylin oil using a small scale homogeniser. Emulsion-forming ability was measured by determination of the volume-surface average particle diameter (d_{32} value) using a Malvern Mastersizer. Emulsion stability was followed for 24 hours, by measuring the turbidity change according to the method of Pearce and Kinsella (1978). For statistical analysis emulsion stability (E_{stab}) was expressed as the turbidity remaining after 24 hours (T_{24}/T_0*100%).

Foam forming and stability

Foam properties were measured as previously described (Chapter 4), using the same hydrolysates as used for determination of the emulsion properties. In summary, foam was prepared with a 0.05% (w/v) supernatant protein solution in 0.02 M imidazole/HCl buffer pH 6.7 containing 3.44 g/l NaCl with a whipping method as described by Caessens et al. (1997). Foam-forming ability (F_0) was defined as the foam volume present two min after starting whipping. Foam stability (Vf_{60}) was expressed as the proportion foam remaining after 60 min relative to foam volume at t =2 min (F_{60}/F_0*100%).

Sensory analysis of bitterness

For assessment of bitterness the supernatant protein solutions were used. Solutions of 5% (w/v) whey hydrolysate or 2% (w/v) casein hydrolysate were prepared. A trained sensory panel, consisting of 7 members, first tasted four concentrations of a standard caffeine solution (0.03, 0.07, 0.11, 0.15% (w/v)) in random order. Subsequently, randomised hydrolysate solutions were judged on bitterness intensity on a five point scale, ranging from not bitter at all (score 0) to extremely bitter (score 5). For statistical analysis the bitterness scores given by the seven panellists were averaged. Whey and casein hydrolysates were tested in separated taste sessions, each session contained a maximum of 6 different hydrolysates. In total, 12 taste sessions were performed. The standard deviation over average scores of caffeine samples over all twelve taste sessions varied from 0.18 to 0.29, depending on the caffeine concentration.

Statistical analysis

Principal Component Analysis (PCA) and Partial Least Square (PLS) techniques were used to study the FTIR spectra (1800 to 800 cm^{-1}) and to study the correlation between the spectra and the functional parameters, using the software package The Unscrambler® (version 7.01, CAMO, Trondheim, Norway). In regression analysis (using Partial Least Squares regression) absorbency values were used as *x*-variables and the functional properties as *y*-variables. The optimum number of principal components (PC) is determined at the point where the residual validation variance reaches its first minimum. The models were validated using leave-one-out cross validation.

RESULTS AND DISCUSSION

Examples of FTIR spectra (1800-800 cm^{-1}) representing differences in hydrolysates prepared from the two protein sources and two classes of enzymes are shown in Figure 1. Figure 1A shows spectra of whey and casein hydrolysates made with Alcalase, as representatives for hydrolysates made with neutral or alkaline enzymes. In Figure 1B spectra of pepsin hydrolysates of whey and casein are shown as representatives of hydrolysates made with acidic enzymes. Comparison of FTIR spectra of whey and casein hydrolysates revealed that absorbencies of whey hydrolysates compared to that of casein hydrolysates are lower in the range of 1700-1485 cm^{-1} and higher from 1170 to 980 cm^{-1}. In addition, the spectra of whey and casein hydrolysates from alkaline and neutral enzymes (Figure 1A) differ in their absorbencies around 1744 cm^{-1}.

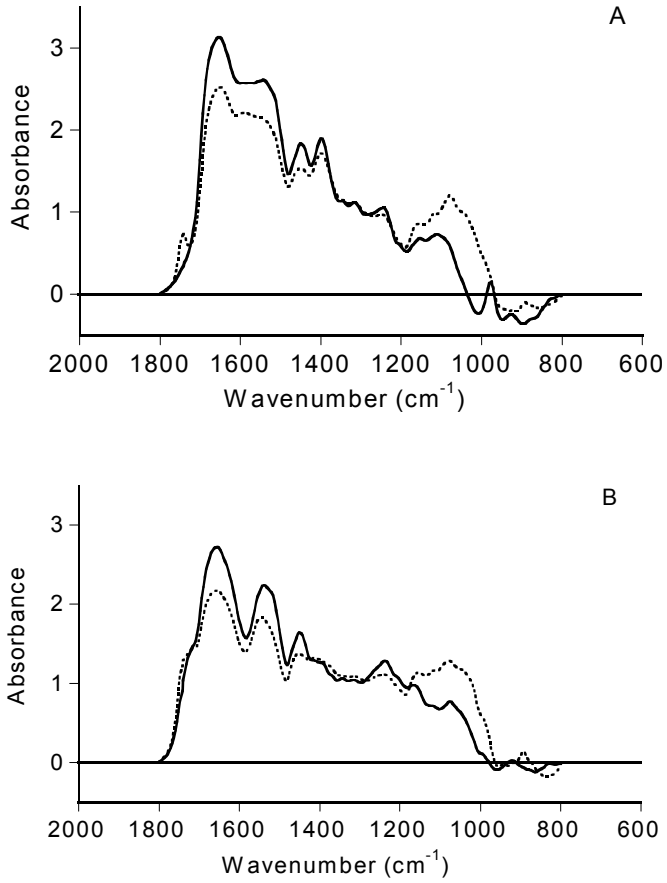

Figure 1 (A+B): Normalised FTIR spectra of casein (Cn, solid line) and whey (Wc, broken line) hydrolysates, representing hydrolysates made with neutral/ alkaline enzymes (1A) and hydrolysates produced with acidic enzymes (1B). 1A: hydrolysates from Alcalase, CnAlc14 (—), WcAlc13 (---). 1B: hydrolysates from Pepsin, CnPep06 (—), WcPep06 (---).

In literature, analysis of protein hydrolysates by FTIR spectroscopy has not been described. Only some studies on FTIR spectra of whey or casein proteins, concerning effects of environmental conditions (pH, temperature, electrolyte balance) on secondary structure, studied by changes of the amide I band, were reported (Casal et al., 1988; Byler & Farrell, 1989; Holt, 1992; Curley et al., 1998). Comparison of our FTIR spectra of hydrolysates with

results concerning proteins presented in literature is difficult, since the protein source is not exactly the same, the measurements conditions differ and data handling of the spectra differs. Spectra of whole proteins were mostly measured in solution, while we measured FTIR spectra in KBr pellets. There is not much known about the consequences of drying on the structure of proteins or peptides. One report was published on the secondary structure of globular proteins in KBr pellets, concluding that the secondary structure was highly conserved upon drying (Forato et al., 1998). However, it is not known whether drying affects the secondary structure of peptides.

Principal component analysis

The first step in statistical data analysis of FTIR spectra was Principal Component Analysis (PCA), used to investigate whether FTIR spectra describe differences in protein hydrolysates properly and whether classes of samples can be distinguished based on these spectra.

The principal component analysis resulted in a model in which the two first principal components explain 95% of the total spectral variation. PC 1 and PC 2 account for 79% and 16%, respectively. A plot of PC 1 versus PC 2 (Figure 2) shows the distribution of the samples on these new, independent variables.

PCA revealed the existence of four clusters, which are defined by protein source, casein or whey, and enzyme source, acidic or neutral/alkaline (Figure 2). In order to assess the influence of protein source and enzyme source separately, PCA was applied to different sets of samples.

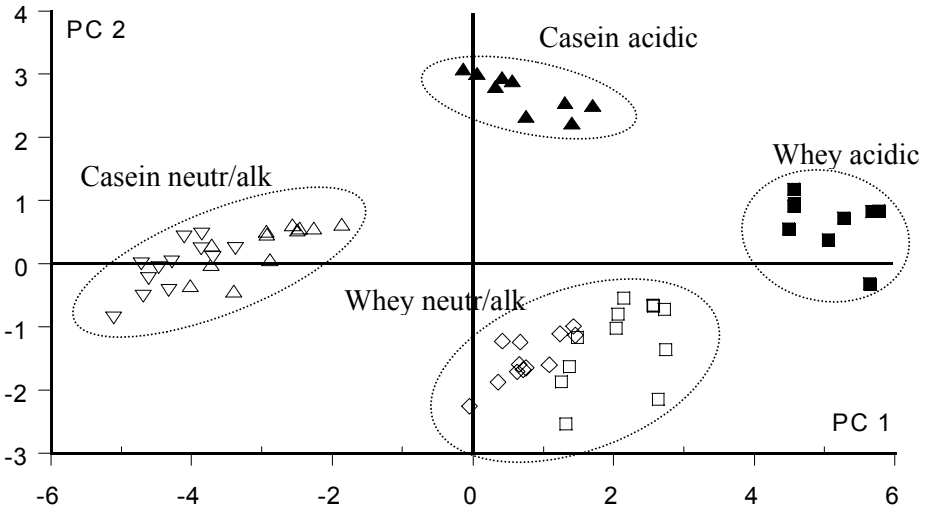

Figure 2: Scores of whey and casein hydrolysates on the first two Principal Components (PC 1 and 2) obtained from principal component analysis of their FTIR spectra. Symbols: casein hydrolysates produced with acidic enzymes (▲), with neutral enzymes (Δ) and with alkaline enzymes (∇). Whey hydrolysates produced with acidic enzymes (■), neutral enzymes (□) and alkaline enzymes (◇). Groups are defined by source of protein (whey/casein) and classes of enzymes: acidic or neutral and alkaline enzymes (neutr/alk).

The plot depicted in Figure 3A represents the result of a PCA upon casein hydrolysates only (N=33). Again, a clear separation between the acidic and the neutral/alkaline samples according to the 1st PC (explained variance 92%) can be noticed. To identify the influence of protein source, PCA was applied to 48 samples obtained with neutral/alkaline enzymes. The result is presented in Figure 3B. A very clear separation of the hydrolysates according to the protein source is obtained. The 1st PC explains 91% of the data variation.

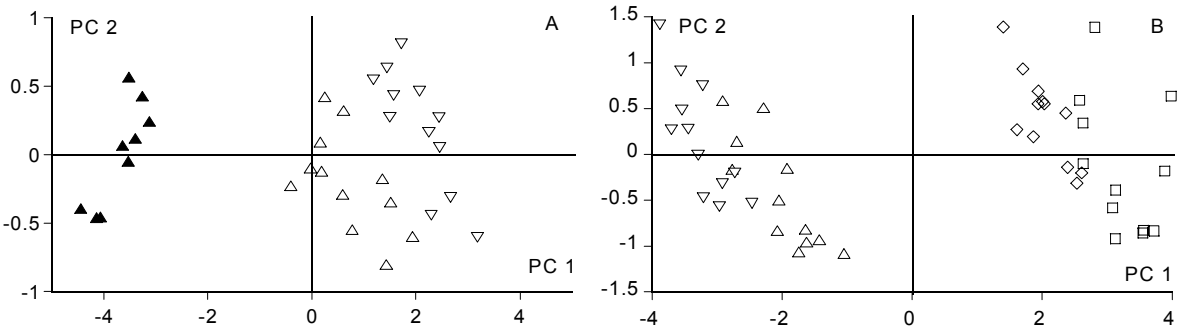

Figure 3 (A+B): Scores of whey and casein hydrolysate samples on the two first PC's obtained from principal component analysis of FTIR spectra of 33 casein hydrolysates (3A) and of 24 whey and 24 casein hydrolysates prepared with neutral and alkaline enzymes (3B). Symbols for casein hydrolysates: acidic (▲), neutral (Δ) and alkaline enzyme hydrolysates (∇). Symbols for whey hydrolysates: neutral (□) and alkaline enzyme hydrolysates (◇).

Principal components are vectors described by linear combinations of the original variables, in our case the absorbencies measured in the infrared spectrum. Loading plots provide information about the contribution of each original variable to a principal component. Variables with high loading weights are responsible for the main variation in the data described by the particular PC. In Figure 4 the loading plot of the 1st PC obtained from the analysis with only casein hydrolysates (Figure 3A) is depicted. From the loading plot the spectral regions responsible for the differences between the acidic and neutral/alkaline casein hydrolysates can be readily identified. Differences between FTIR spectra of these classes of hydrolysates were mainly observed between 1743 to 1705 cm^{-1}, around 1585 cm^{-1} and around 1400 cm^{-1}. These frequencies are associated to carboxylate ion stretching vibrations (1650-1550, near 1400 cm^{-1}) (Byler & Farrell, 1989; Silverstein et al., 1991), carboxylic acid dimers (1720-1700 cm^{-1}) (Willard et al., 1981; Silverstein et al., 1991) and monomers of saturated aliphatic acids (near 1760 cm^{-1}) (Silverstein et al., 1991). Therefore, differences in the spectra might be related to the presence of ionised or non-ionised carboxyl groups caused by different pH values of hydrolysates.

To elucidate whether pH differences of protein hydrolysates are responsible for differences in FTIR spectra from hydrolysates of the different enzyme classes, some samples prepared with acidic enzymes were neutralised after enzyme treatment and lyophilized. FTIR analyses showed little or no difference with the samples measured before, demonstrating that differences as observed in figure 3A are not caused by pH differences.

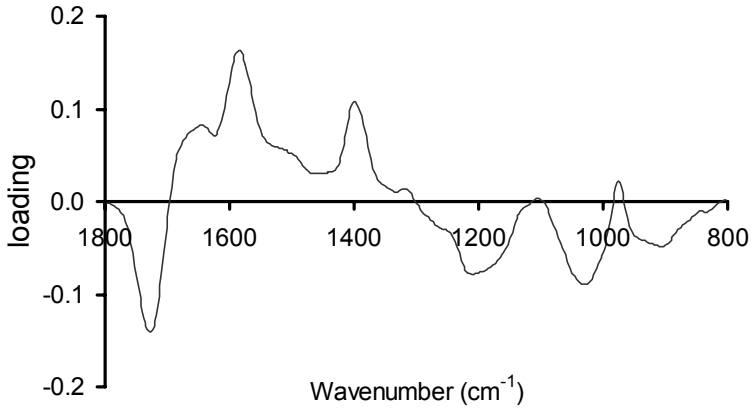

Figure 4: Loading plot of 1st PC obtained after principal component analysis of FTIR spectra of casein hydrolysates.

From the PCA with FTIR spectra, it can be concluded that the infrared spectra effectively differentiate between hydrolysates made from different protein sources and enzyme classes. Previously, size exclusion chromatography (SEC) and reversed phase chromatography (RPC) have been used to characterise the 33 whey and 33 casein hydrolysates (Chapter 2). PCA with these chromatograms did not result in such a clear grouping of the hydrolysates as is found now. From these observations it can be concluded that, so far, FTIR spectroscopy is the only characterisation method that can distinguish fully between hydrolysates made from different protein sources and different classes of enzymes.

Prediction of functional properties from FTIR spectra

As FTIR spectroscopy is capable of describing differences between protein hydrolysates effectively, it is interesting to investigate whether the FTIR spectra can be related to functional properties of these hydrolysates. For this purpose, multivariate regression analysis (PLS regression) was performed.

PCA based on FTIR data revealed differences between samples according to protein and enzyme source. Therefore, regression analysis was performed for six different sample sets, categorised on basis of protein and enzyme source. Sample set 1 contains whey hydrolysates prepared with all eleven different enzymes, sample set 2 contains casein hydrolysates from all eleven enzymes and sample set 3 contains all samples from sets 1 and 2. Sample set 4 contains whey hydrolysates produced with neutral and alkaline enzymes (8 enzymes), sample set 5 contains casein hydrolysates from those 8 enzymes and sample set 6 contains all samples from sets 4 and 5. The number of samples used for regression analysis depends on the number of samples for which the functionality is determined (all samples for bitterness and solubility, a random selection of samples for emulsion and foam properties). Outliers (influential samples due to high leverage and/or high residuals) were removed to obtain optimal regression models. In Table 2 the minimum and maximum values for each functional property, regarding the samples included in the regression analysis, are presented as well as the results of the regression analyses. In the following, the results will be separately discussed for the various functional properties of the hydrolysates.

Table 2: Results of PLS regression with FTIR spectra as independent variables and various hydrolysate functional characteristics as dependent variable.

Functional property	Range[a]	PC's[b]	R^2 [c]	R^2 CV[d]	bias[e]	SEP[f]
Hydrolysates from all enzyme sources (sample sets 1-3)						
Bitterness (score from 0-5)						
Whey (25)[g]	0.9 - 2.3	3	0.75	0.62	-0.008	0.29
Casein (31)	1.4 - 3.9	4	0.78	0.60	0.026	0.48
Whey + casein (59)	0.9 - 3.8	3	0.74	0.69	0.002	0.45
Solubility (% w/w)						
Whey (32)	18 - 96	2	0.86	0.82	0.011	8.78
Casein (31)	42 - 85	4	0.86	0.76	-0.094	6.93
Whey + casein (66)	18 - 96	6	0.87	0.76	-0.033	8.73
Emulsion forming (d_{32}, μm)[h]						
Whey (20)	0.6 - 5.3	3	0.91	0.83	-0.059	0.66
Casein (16)	0.5 - 4.6	2	0.94	0.89	-0.021	0.54
Whey + casein (37)	0.5 - 5.3	4	0.86	0.77	-0.009	0.75
Emulsion stability (E_{stab}, %)[i]						
Whey (19)	56 - 100	4	0.93	0.86	0.36	5.47
Casein (16)	5 - 100	2	0.93	0.89	0.53	13.4
Whey + casein (36)	5 - 100	4	0.91	0.86	-0.23	11.3
Foam forming (F_0, ml)[j]						
Whey (17)	0 - 46	4	0.94	0.87	0.37	6.04
Casein (19)	33 - 64	nd[l]				
Whey + casein (39)	0 - 64	4	0.85	0.75	0.19	10.5
Foam stability (Vf_{60}, %)[k]						
Whey (20)	0 - 72	5	0.76	0.68	-2.61	15.7
Casein (18)	18 - 82	1	0.84	0.84	-0.014	13.5
Whey + casein (23)	0 - 82	1	0.83	0.82	-0.33	13.7
Hydrolysates from neutral/alkaline enzymes (sample sets 4-6)						
Bitterness (score from 0-5)						
Whey (20)	0.9 - 2.4	1	0.76	0.74	0.0015	0.26
Casein (20)	1.3 - 3.5	4	0.85	0.76	-0.010	0.35
Whey + casein (43)	0.9 - 3.5	5	0.89	0.79	0.0015	0.38
Solubility (% w/w)						
Whey (23)	38 - 96	4	0.90	0.80	-0.21	7.60
Casein (21)	47 - 85	4	0.96	0.92	0.16	3.66
Whey + casein (46)	38 - 96	5	0.89	0.81	-0.019	6.08
Emulsion forming (d_{32}, μm)						
Whey (14)	0.6 - 3.5	3	0.91	0.82	0.055	0.45
Casein (12)	0.5 - 3.6	nd				
Whey + casein (24)	0.5 - 3.1	4	0.86	0.71	-0.027	0.43
Emulsion stability (E_{stab}, %)						
Whey (13)	56 - 95	3	0.92	0.88	-0.20	5.28
Casein (11)	5 - 98	2	0.96	0.92	0.37	11.6
Whey + casein (25)	5 - 98	3	0.91	0.87	0.077	11.8
Foam forming (F_0, ml)						
Whey (13)	0 - 46	3	0.93	0.81	-0.49	7.72
Casein (12)	33 - 63	nd				
Whey + casein (25)	0 - 63	2	0.86	0.83	0.041	7.66
Foam stability (Vf_{60}, %)						
Whey (15)	0 - 72	4	0.87	0.73	-0.82	16.2
Casein	20 - 59	nd				
Whey + casein	0 - 72	1	0.78	0.75	0.22	14.2

[a]Lowest and highest value for samples used in regression analysis. [b]Number of principal components used in regression analysis. [c]Multiple correlation coefficient (coefficient of determination) for calibration samples. [d]Multiple correlation coefficient of the cross validation. [e]Average difference between predicted and measured values. [f]Standard error of prediction. [g]Number of samples used for regression analysis. [h]Volume surface average particle diameter of emulsion droplets. [I]Emulsion turbidity after 24 hours relative to turbidity at t=0. [j] Initial foam volume. [k]Foam volume after 60 min relative to initial foam volume. [l]nd: regression parameters were not determined because $R^2 < 0.5$.

Bitterness

The whey hydrolysate concentration used in sensory analysis was 2.5 times higher than the casein hydrolysate concentration, since casein is known to yield more bitter hydrolysates than whey protein (Petrischek et al., 1972; Lemieux & Simard, 1992). Despite the lower concentration of the casein hydrolysates, their perceived bitterness scores were generally higher than those for whey hydrolysates (Table 2).

PLS regression analysis with sample sets 1 to 3 resulted in models having explained variances ≤69% (R^2CV ≤0.69) (Table 2). The correlation coefficient between predicted and measured values is best for the model containing both casein and whey hydrolysates, since the combination of samples results in a broader range of bitterness values. Exclusion of samples produced with enzymes active at low pH results in improved bitterness prediction (sample sets 4-6; Table 2). The hydrolysates produced at low pH values were neutralised before sensory testing, to mask the acid taste. As a consequence these samples were rather salty which might have influenced the bitterness perception. Figure 5 shows the correlation between predicted and measured bitterness scores of whey and casein hydrolysates made with neutral and alkaline enzymes. The correlation (R^2CV) between predicted and measured values is 0.79.

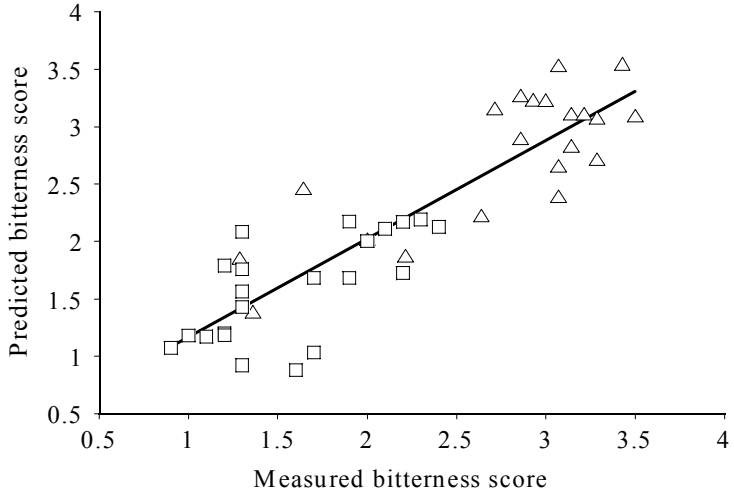

Figure 5: Correlation between predicted (obtained from FTIR spectra) and measured bitterness scores for casein (Δ) and whey (□) hydrolysates produced with neutral/alkaline enzymes. $R^2CV = 0.79$.

The regression analyses resulted in bitterness predictions with a coefficient of determination (R^2CV) that ranged between 0.60 and 0.79 for the different sample sets, which is rather good compared to prediction of sensory properties reported in literature, which mainly concerns studies with cheese flavour. One study used near infrared spectra to predict flavour attributes of cheese, which resulted in R^2 values from 0.27 to 0.59 (Sorensen & Jepsen, 1998). Other studies used chemical parameters or chromatography to study flavours of cheese. To predict bitterness of cheese reversed phase chromatography data were used in combination with chemical parameters, like content of intact casein, ratio between ultrafiltration fractions and a

ratio between hydrophobic and hydrophilic peptides. The regression analysis resulted in explained variance for bitterness of 95% for a set of 6 samples and of only 59% for a set of 19 samples (Frister et al., 2000). For Swiss cheese, Vangtal and Hammond (Vangtal & Hammond, 1986) reported a correlation coefficient (R^2) of 0.53 between bitterness and degree of proteolysis. Moreover, they used factor analysis to define flavour factors describing various taste attributes and to define chemical factors describing the chemical composition based on *e.a.* fatty acid analysis. The correlation between the flavour factors and chemical factors varied between R^2 0.63 to 0.85, which is comparable to the correlation between the FTIR spectra and bitterness in the present study, however, determination of chemical parameters is much more laborious.

Solubility

Solubility of whey hydrolysates varied between 18 and 96%, while the minimum solubility of casein hydrolysates was 42%. Acidic enzyme hydrolysates generally showed lower solubility values than the neutral/alkaline enzyme hydrolysates. Solubility prediction of the whey and casein hydrolysates from FTIR data is reasonably good. For casein hydrolysates the prediction improves if acidic enzyme hydrolysates are excluded from analysis, which is not observed for whey hydrolysates. The acidic enzyme hydrolysates of casein seemed to influence the prediction model of casein solubility more than did the acidic enzyme hydrolysates of whey in case of the whey solubility model.

Emulsion forming

Emulsion-forming ability of casein hydrolysates was modelled excluding samples with very high d_{32} values (> 7 μm), since these samples have a high leverage and/or high residual variance on the model, which makes them influential samples. Regression analysis with the remaining casein hydrolysates (N=16) resulted in prediction with $R^2CV = 0.89$. However, the prediction was mainly based on the difference between two groups: emulsions with relative high d_{32} values (3 to 5 μm), opposed to emulsions with low average particle sizes (0.5 to 2.0 μm). The emulsions with high d_{32} were prepared with acidic enzymes. If these samples are excluded from the analysis the variation range of d_{32} values is rather small and the differences between samples can not be explained on basis of the FTIR spectra.

Emulsion-forming properties of whey hydrolysates are predicted rather good, the total explained variance is 83%. Exclusion of the acidic enzyme hydrolysates (sample set 4) results in similar prediction. The range of variation was probably still large enough to achieve good prediction. Analysis of casein and whey hydrolysates together results in models with explained variance of 77% and 71% for sample set 3 and samples set 6, respectively. In Figure 6 the result obtained with sample set 6 is presented.

Emulsion-forming ability of protein hydrolysates was not predicted before, although, some articles were published about the prediction of emulsion-forming ability of proteins, using the emulsifying activity index (EAI) as emulsion parameter, which is calculated from the

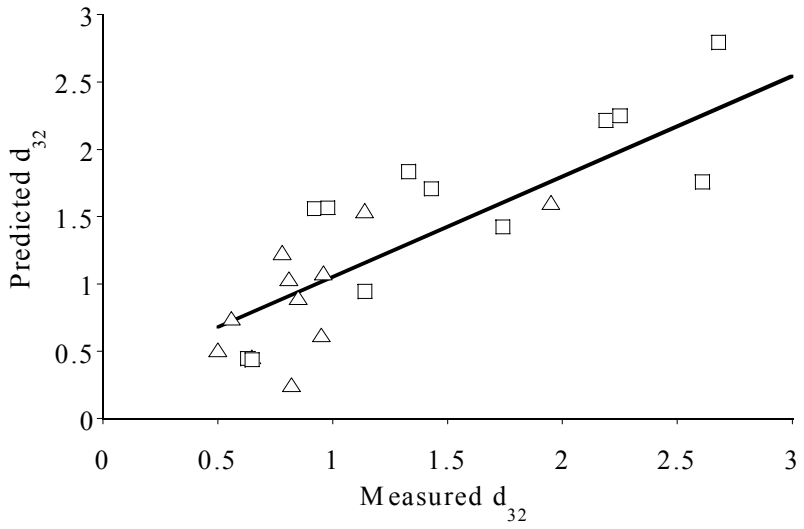

Figure 6: Prediction of emulsion-forming ability of casein (Δ) and whey (□) hydrolysates. Prediction was based on FTIR spectra from hydrolysates prepared with neutral and alkaline enzymes. R^2 CV = 0.71.

turbidity of emulsions (Pearce & Kinsella, 1978) instead of the d_{32} value. The EAI of protein emulsions has been correlated to hydrophobicity of proteins, with reported correlation coefficients (R^2) between measured and predicted values of 0.71 (Kato & Nakai, 1980), 0.58 (Voutsinas et al., 1983) and 0.44 (Nakai et al., 1980). Emulsion activity index has also been correlated to a set of 18 physicochemical proteins parameters (*e.g.* % α-helix, hydrophobicity, charge density, binding of fluorescent probes), which resulted in a multiple correlation coefficient between predicted and measured values of 0.75 (Arteaga & Nakai, 1993). Spectral properties of proteins like UV-absorption and fluorescence were correlated to emulsion-forming properties in a study on whey-pea and whey-potato protein mixtures, resulting in a multiple correlation coefficient (R^2) of 0.79 (Jackman & Yada, 1990).

Emulsion stability

Prediction of stability of whey and casein hydrolysates emulsions is quite good (Table 2). The accuracy of prediction is comparable to the prediction of emulsion stability using molecular weight distribution (Chapter 3). MWD can be predicted as well on basis of FTIR spectra (data not shown).

In literature some relations between emulsion stability and chemical characteristics of proteins for protein stabilised emulsions are described. Stability of emulsions prepared with combinations of whey and pea proteins was correlated to hydrophobicity and spectral properties like UV absorption, resulting in a multiple correlation coefficient (R^2) of 0.69. In case of a combination of whey and potato proteins the correlation between predicted (based on fluorescence and UV measurements) and measured values appeared to be poor (R^2 = 0.47) (Jackman & Yada, 1990). Hydrophobicity of proteins was, apart from emulsifying activity, also correlated to emulsion stability (R^2 = 0.58) (Voutsinas et al., 1983).

Foam forming

Foam-forming ability of whey hydrolysates could be modelled satisfactorily, whereas for casein hydrolysate foams no correlation between FTIR spectra and foam-forming ability was found. Foam-forming ability of casein hydrolysates was high in most samples, resulting in only small differences between values measured for foam-forming ability. However, if the data of casein and whey hydrolysates are combined, the variation between foam-forming ability of samples is sufficiently large to obtain a good model ($R^2CV = 0.75$). The whey hydrolysate model without the acidic enzyme hydrolysates is not as good as the model with all samples (sample set 4 compared to sample sets 1, Table 2). The foam-forming ability of acidic whey hydrolysates does not differ systematically from that of the other enzyme hydrolysates. Therefore, the less accurate prediction probably comes from the lower number of samples used for prediction.

Arteaga and Nakai (1993) predicted foam-forming capacity of proteins using the same chemical parameters as used to predict emulsion properties, which resulted in a correlation coefficient (R^2) of predicted versus measured values of 0.70. Foam capacities of 16 proteins were correlated to hydrophobicity of denatured protein in conjunction with viscosity and resulted in a correlation coefficient (R^2) of 0.78 (Townsend & Nakai, 1983).

Foam stability

Regression analysis of all whey hydrolysates results in poor correlation between FTIR spectra and foam stability. The correlation obtained with sample set 4, which excludes acidic enzyme hydrolysates, is only slightly better. Only six of the 22 whey hydrolysates were able to form stable foams, the foam of all other hydrolysates disappeared within one hour. There are insufficient samples with stabilising ability to attain a good prediction model for foam stability in case of whey hydrolysates.

Foam stability of casein hydrolysates is predicted rather well, as was expected from the prediction on basis of the MWD (Chapter 4). If the acidic enzyme hydrolysates are excluded it is not possible to obtain a good prediction. A relatively large part of the remaining hydrolysates is not able to stabilise the hydrolysate. Therefore, the spread in results is no longer large enough to result in good correlations.

In conclusion, the combination of FTIR spectroscopy and multivariate data analysis proved very valuable in protein hydrolysate characterisation. FTIR spectra appear to correlate to various functional properties of whey and casein hydrolysates, some of which could not be predicted from other analytical methods until now. FTIR spectroscopy is not laborious and might substitute labour intensive functionality measurements.

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CHAPTER 6

Optimisation of the Angiotensin Converting Enzyme inhibiting activity of whey protein hydrolysates using response surface methodology.

ABSTRACT

Protein hydrolysates inhibiting the Angiotensin Converting Enzyme (ACE*) in vitro*, are potentially interesting constituents for blood pressure decreasing products. To minimise the amount of hydrolysate needed, the ACE-inhibiting activity should be maximised. The total peptide composition of a hydrolysate determines its ACE-inhibiting ability and depends on the specificity of the proteolytic enzyme and the process conditions used for the production of the hydrolysate. In the present research, the effect of process conditions on the ACE-inhibiting activity of whey protein concentrate hydrolysed with a pancreatic enzyme mixture was investigated systematically using response surface methodology. It was shown that the obtained model effectively predicted the ACE-inhibiting activity. Hydrolysis conditions for optimal ACE-inhibiting activity were defined using the response surface model.

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INTRODUCTION

Proteins are an important source of energy and essential amino acids in the human diet. For some food applications proteins should be hydrolysed, for example for use in hypoallergenic products or in clinical nutrition (Clemente, 2000). Proteins may also be hydrolysed with the purpose to produce biologically active peptides, which can be added to foods as part of a complete hydrolysate or as (partly) purified peptides. Milk protein hydrolysates for example contain several bioactive peptides, including antihypertensive peptides (Meisel & Schlimme, 1996; Xu, 1998; Clare & Swaisgood, 2000). The antihypertensive effect of peptides is related to the inhibition of the Angiotensin Converting Enzyme (ACE). ACE activity results in blood pressure increase via conversion of angiotensin I to angiotensin II, which is a vasoconstrictive peptide, and via degradation of bradykinin, which is a vasodilatative peptide. Inhibition of ACE, *e.g.* by peptides, results in blood pressure decrease (Meisel & Schlimme, 1996; Clare & Swaisgood, 2000). Numerous potential ACE-inhibiting peptides, deduced from *in vitro* activity measurements, have been reported in literature (Yamamoto, 1997; Abubakar et al., 1998; FitzGerald & Meisel, 1999; Fujita & Yoshikawa, 1999; Pihlanto Leppala et al., 2000; Kim et al., 2001). Various studies describe the purification and the identification of ACE-inhibiting peptides from hydrolysates using a range of chromatographic techniques (Maruyama & Suzuki, 1982; Miyoshi et al., 1991; Lee et al., 1999; Matsui et al., 1999). From these reports, it appears that ACE-inhibiting peptides are present in many fractions obtained with columns based on different separation principles, indicating that the molecular properties of ACE inhibitors are rather diverse. The fact that ACE-inhibiting activity is found in hydrolysates produced with different proteins, proteases and hydrolysis conditions also indicates that a variety of peptides is able to inhibit ACE. As ACE inhibition is this a-specific, chromatographic purification of hydrolysates will always result in the loss of part of the active peptides. Therefore, purification methods are not suited for economic production of an ACE-inhibiting product.

If there is any general feature of ACE-inhibiting peptides, it is their molecular weight. Most of the reported peptides exhibiting ACE-inhibiting activity are low molecular weight peptides (generally <12 amino acids). Therefore, some selection of active peptides can be achieved by ultrafiltration, resulting in the production of a permeate with increased ACE-inhibiting activity compared to the parental hydrolysate (Mullally et al., 1997; Pihlanto Leppala et al., 2000; Kim et al., 2001). However, with ultrafiltration, part of the protein material is lost and an extra processing step is introduced.

As stated before, many peptides exhibit ACE-inhibiting activity. The combination of all these peptides determines the ACE-inhibiting activity of the hydrolysate. During hydrolysis, ACE-inhibiting peptides are continuously formed and degraded again. Maximum ACE-inhibiting activity of the hydrolysate is a result of an optimum between these two processes. To optimise the overall peptide composition of hydrolysates, the hydrolysis process has to be investigated. Process optimisation preserves the nutritional value of the protein and is advantageous because of the low costs.

The peptide composition of hydrolysates depends on the specificity of the proteolytic enzyme and on process conditions. The effects of enzyme source, enzyme concentration and

hydrolysis time on ACE-inhibiting activity of hydrolysates have been described in literature (Matsui et al., 1993; Mullally et al., 1997; Abubakar et al., 1998; Pihlanto Leppala et al., 2000; Kim et al., 2001). For a complete optimisation of the hydrolysis process the influence of other parameters, like pH and temperature of hydrolysis, and the interactive effects between hydrolysis parameters on ACE-inhibiting activity should also be regarded. Response surface modelling has been proven to be a valuable tool for simultaneous optimisation of several process parameters for various processes, like fermentation processes, hydrolysis processes and chemical reactions (Baek & Cadwallader, 1995; Diniz & Martin, 1996; Ibanoglu et al., 1998; Kimmel et al., 1998; Liu et al., 2000; Wu et al., 2000).

In the present study, whey protein concentrate was hydrolysed with Corolase PP, a pancreatic enzyme mixture that was shown to yield hydrolysates with rather good ACE-inhibiting activity (Mullally et al., 1997). The effects of five process parameters on ACE-inhibiting activity were studied with a central composite design as described by Cochran and Cox (1957). The five studied parameters comprise the pre-treatment temperature of the protein and four hydrolysis parameters, *i.e.* pH, temperature, hydrolysis time and enzyme to substrate ratio.

MATERIALS AND METHODS

Whey protein concentrate (WPC 60) was obtained from Milei GmbH (Stuttgart, Germany). ACE from rabbit lung and FAPGG were purchased by Sigma. Corolase PP was supplied by Röhm Enzyme GmbH (Darmstadt, Germany).

Whey hydrolysis

Whey protein was hydrolysed with Corolase PP in a pH-Stat set up (Titrino 718, Metrohm) according to the hydrolysis conditions defined by the experimental design. The whey protein concentration was fixed at 5% (w/v) on protein basis. Prior to hydrolysis the protein suspension was stirred during 15 min at the 'pre-treatment temperature'. The hydrolysis process was terminated by heating the solution at 90°C during 15 min. Hydrolysates were freeze-dried without further separation.

In the experimental design five hydrolysis parameters (k = 5) were varied; pre-treatment (preT) temperature of the protein suspension, enzyme to substrate ratio (E/S), pH, temperature (Temp) and time of hydrolysis. The process parameters were optimised using a central composite rotatable design (CCRD), containing five levels for each process parameter, coded as $-\alpha$, -1, 0, +1, $+\alpha$ (Table 1). The design exists of three experiment categories: centre experiments, having all parameters set at level 0 (repeated several times to determine reproducibility, 6 experiments in the currently used design); cube experiments, which are combinations of –1 and +1 levels (2^k experiments for a full factorial design); and star experiments, with one parameter at an extreme value ($-\alpha$ or $+\alpha$) and the other parameters at level 0 (2 x k experiments) (Myers & Montgomery, 1995). The experiments were performed in three phases. At first a fractional factorial design was made (consisting of 16 cube samples and 3 centre samples). Subsequently, the design was extended to a full factorial design (16

cube samples plus 1 centre sample), followed by production of the star experiments (10 samples) and two additional centre samples. In total, the central composite design consisted of a full factorial design plus star experiments and 6 centre experiments, resulting in 48 experiments.

Table 1: Coded and uncoded settings of the process parameters for whey protein hydrolysis, according to a central composite design.

	Level				
Process parameter	-2.38 (-α)	-1	0	1	2.38 (α)
pretreatment T (°C)	17	45	65	85	100 (113)[a]
E/S (% w/w)	0 (-0.19)	0.5	1	1.5	2.19
pH	4.78	6.5	7.75	9.0	10.72
Temperature (°C)	21	35	45	55	69
Hydrolysis time (hours)	0 (-1.6)	0.5	2	3.5	5.57

[a] Values within brackets: design value for α or - α, which can not be applied practically.

Protein determination

Protein concentration was measured by determination of total nitrogen on an N-analyser (NA 2100 Protein, CE instruments, Interscience, Breda, the Netherlands). For calculation of protein concentrations a Kjeldahl factor of 6.38 was used.

Degree of hydrolysis

The degree of hydrolysis (DH) was measured spectrophotometrically according to the method of Adler-Nissen (1979) adapted for use in microtiter 96-wells plates Hydrolysate samples were diluted in 1% (w/v) SDS to a concentration of 0.05% (w/v on protein basis). The starting material (protein solution) was diluted to 0.1% (w/v on protein basis). A leucine concentration range was used as standard to calculate the amount of free amino groups. Sample solutions (15 µl) were 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 hour in a 50°C stove. The reaction was stopped by addition of 90 µl of 0.1 M HCl. Subsequently, absorption at 340 nm was measured with a Packard Spectra Count plate reader (Packard Instruments B.V., Groningen, The Netherlands).

ACE inhibition measurements

ACE activity was measured spectrophotometrically in 96-wells plates using 2-furanacryloyl-l-phenylalanylglycylglycine (FAPGG) as substrate, based on the assay described by Bunning and co-workers (1983). The enzyme assay was performed at 37 °C in 50 mM Hepes buffer, pH 7.5 containing 300 mM NaCl, 1.5 mM FAPGG and 16 mU ACE/ml. By definition, one unit of ACE produces 1 µmol hippuric acid from Hippuryl-His-Leu per min in 50 mM Hepes and 300 mM NaCl at pH 8.3 at 37 °C (Sigma). The enzyme activity was measured in a temperature controlled plate reader (Ultramark, Biorad, Veenendaal, the Netherlands) following the absorption decrease at 340 nm during 15 min. Subsequently, the absorption decrease per min (v) was calculated as a measure for ACE activity. The ACE activity in the

presence of five concentrations of hydrolysate protein, ranging from 0.05 to 0.8 mg/ml was determined. The relation between enzyme activity and inhibitor concentration ([I]) was linearised by plotting $1/v$ versus [I]. These plots were used to calculate the IC_{50} value, which is the amount of hydrolysate needed to inhibit 50% of the enzyme activity.

Statistical analysis

Statistical analysis was performed with The Unscrambler® (Camo, Camo ASA, Oslo, Norway).

For two response parameters, DH and ACE-inhibiting activity, the population standard deviation, which is the deviation from the mean over all samples, and the standard deviation over repeated centre samples were calculated. The standard deviation (SD) is calculated according to equation 1, with m = total number of experiments for the population SD and m = p -1 for the SD over centre samples, with p = the number of centre samples.

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{m}} \qquad [1]$$

For response surface analysis the main variables were centred before analysis and the interaction and square effects were calculated from standardised main variables (weighted (1/SD) and centred). The centre value for each main variable was defined as the mean value of the parameter over all samples included in the analysis ($(\Sigma x_i)/n$). Quadratic response surface analysis was based on multiple linear regression taking into account the main, the quadratic and the interaction effects, according to equation 2. The significance of the b-coefficients calculated by regression analysis was tested with the student-t test.

$$Y = b_0 + \sum_{i=1}^{5} b_i X_i + \sum_{i=1}^{5} b_{ii} X_i^2 + \sum_{i<j=2}^{5} b_{ij} X_i X_j + e \qquad [2]$$

Analysis of variance (ANOVA) was performed on the models calculated with linear regression. The R^2 value, the residual error, the pure error (calculated from the repeated measurements) and the lack of fit (l.o.f.) were calculated. The lack of fit indicates whether the calculated response surface represents the true surface. The sum of squares (SS) of the lack of fit is calculated as $SS_{residual} - SS_{pure\ error}$. The significance of the lack of fit is tested with an F-test with $F=MS_{lof}/MS_{pure\ error}$, with MS being the mean sum of squares (SS/df) (Ostle & Malone, 1988; Myers & Montgomery, 1995).

RESULTS AND DISCUSSION

Degree of hydrolysis and ACE inhibition, general statistics

The parameter settings for the production of whey hydrolysates were chosen with the aim to obtain hydrolysates with a large variation in degree of hydrolysis. Therefore, the lower extreme values ($-\alpha$) for hydrolysis time and enzyme concentration were defined as zero (Table 1). Consequently, two samples consist of non-hydrolysed whey protein. The maximum ACE-inhibiting activity reached with these samples was 45%, which means that no IC_{50} values could be calculated. For statistical calculations on ACE-inhibiting activity these samples were excluded. The results of the hydrolysis experiments are summarised in Table 2,

giving the range of variation, the average values and the population standard deviation over 46 samples for the ACE-inhibiting activity and over 48 samples for the degree of hydrolysis (DH). The values for the centre samples are specified separately since these samples are the only replicates, providing an indication about the reproducibility of the hydrolysis process. The population standard deviation is calculated to judge whether it is likely that the response parameter is influenced by the process conditions. A high population standard deviation indicates that the differences are probably due to variation in the process parameters and not due to coincidence.

Table 2: Summarised statistics for ACE-inhibiting activity and DH of whey hydrolysates produced according to the central composite design.

		All samples	Centre samples
IC_{50} (mg/ml)[a]	Range	0.16 - 0.84	0.17 - 0.21
	Average	0.28	0.19
	SD[b]	0.12	0.015
DH[c]	Range	0 - 35	10 - 14
	Average	12	12
	SD[b]	9.0	1.3

[a] Concentration of hydrolysate protein needed to inhibit ACE activity by 50%.
[b] Standard deviation; calculation as outlined in materials & methods section.
[c] Degree of hydrolysis.

The DH values of the centre samples varied between 10 and 14%, with an average and SD of 12 ± 1.3%. Other researchers, who calculated DH from the NaOH consumption during the pH-stat experiment, reported relative standard deviations of 5% to 22% (Behnke et al., 1989), 3% to 6% (Hung et al., 1984) and 5% to 11% (Galvao et al., 2001), which is comparable to our results. The average IC_{50} value of the centre samples was 0.19 ± 0.015 mg/ml (Table 3). The relative standard deviation is 7.9%, which is rather good with respect to the variability of the DH values. Reproducibility of ACE activity measurements is rarely mentioned in literature. Only a few articles concerning ACE activity measurements in blood sera were found, reporting deviations of 2% to 10%, depending on *e.a.* ACE activity levels (Harjanne, 1984; Maguire & Price, 1985). In literature reporting on ACE-inhibiting activity of peptides or protein hydrolysates, the reproducibility of the determination of ACE inhibition is not mentioned at all.

The ACE-inhibiting activity of all whey hydrolysates varied between IC_{50} values of 0.17 to 0.88 mg/ml, whereas the IC_{50} value of ~75% of the hydrolysates was <0.31 mg/ml. The hydrolysates with IC_{50} values >0.31 mg/ml were generally low-DH hydrolysates (DH <10%), whereas the hydrolysate displaying the highest ACE-inhibiting activity was hydrolysed to a DH of 30%. These data suggest that extensive hydrolysis, resulting in the formation of many low molecular weight peptides, results in high ACE-inhibiting activity. This seems to agree with the fact that most reported ACE-inhibiting peptides are low molecular weight peptides. Moreover, an increase in enzyme concentration and/or hydrolysis time was shown to result in enhanced ACE-inhibiting activity of fish protein hydrolysates (Matsui et al., 1993). However,

other studies concerning ACE inhibition by protein hydrolysates prepared with various enzymes, showed that ACE-inhibiting activity may also decrease with prolonged hydrolysis, suggesting that initially produced ACE-inhibiting peptides were subsequently degraded (Lee et al., 1999; Kim et al., 2001).

Response surface modelling

The population standard deviation for ACE-inhibiting activity over all protein hydrolysates is considerably higher than the standard deviation over the repeated centre samples. Therefore, process parameters probably influence ACE inhibition by hydrolysates, which makes regression analysis interesting. Quadratic response surfaces and the regression coefficients provide information about the effects of individual process parameters and of their interactive effects on ACE-inhibiting activity. Moreover, process parameters needed to reach maximum ACE-inhibiting activity can be estimated.

In the experimental design five parameters were varied, which implies that 21 *b*-coefficients had to be estimated, *i.e.* the coefficients for 5 main effects, 5 quadratic effects, 10 interactions and 1 constant. One sample with a markedly high IC_{50} value (0.84 mg/ml, compared to IC_{50} values <0.57 mg/ml for the other samples), differed strongly from the rest of the samples and was, therefore, considered as an outlier. This sample and the two non-hydrolysed samples that did not reach 50% ACE inhibition were excluded from regression analysis.

Regression coefficients and overall performance of two response surface models are shown in Table 3. The initial regression analysis performed with all regression parameters (‘complete model’) revealed that several terms were not significant (p-value >0.2). In order to simplify the model, non-significant terms were eliminated step by step from the regression model, starting with quadratic terms and interaction effects, ensuring that the R^2 of the model does not change significantly (backward elimination). This procedure resulted in a model with 14 regression terms (Table 3; 'simplified model').

The R^2 for this ‘simplified model’ is 0.816 which is similar to that of the complete model (R^2 = 0.822). The lack of fit of the model (Table 3) indicates whether the calculated response surface represents the true shape of the surface. The lack of fit is significant in both models (F-values of the complete and simplified models are 16 and 12 respectively). However, the R^2 value is high, indicating that the models are well adapted to the responses. Possibly, higher order terms are needed to describe the true surface, or a variable influencing the responses, such as a block effect due to the order in which the experiments were performed, is not included in the model. In addition, the lack of fit might result from a low standard deviation of the ACE-inhibiting activity of the centre samples compared to the standard deviation of the ACE inhibition measurement at other points in the response surface. Nonetheless, the ACE-inhibiting activity is predicted rather good, as is shown by the correlation coefficient (R^2) of 0.82. Therefore, the model is suitable to predict ACE-inhibiting activity of new samples.

The regression coefficients and the response surfaces were used to study the effects of various parameters on the ACE-inhibiting activity. The *b*-coefficients for time and enzyme to substrate ratio (E/S) have negative signs, so both an increase in time and an increase in E/S result in a reduction of IC_{50} value, hence, increased ACE-inhibiting activity. The influence of

Table 3: Regression coefficients and their p-values for the linear regression model for prediction of ACE inhibition by whey protein hydrolysates.

	Complete model		Simplified model	
	b-coefficient	p-value	b-coefficient	p-value
Intercept	0.19	0.000	0.19	0.000
pH	$-2.36*10^{-2}$	0.001	$-2.34*10^{-2}$	0.000
Temp	$-0.19*10^{-2}$	0.038	$-0.20*10^{-2}$	0.017
Time	$-1.86*10^{-2}$	0.004	$-1.77*10^{-2}$	0.002
preT [a]	$-0.02*10^{-2}$	0.516	$-0.03*10^{-2}$	0.488
E/S [b]	$-3.58*10^{-2}$	0.052	$-3.71*10^{-2}$	0.021
pH*Temp	$0.03*10^{-2}$	0.969		
pH*Time	$1.17*10^{-2}$	0.176	$1.17*10^{-2}$	0.129
pH*preT	$1.13*10^{-2}$	0.208	$1.13*10^{-2}$	0.157
pH*E/S	$-0.34*10^{-2}$	0.691		
Temp*Time	$0.39*10^{-2}$	0.625		
Temp*preT	$-1.47*10^{-2}$	0.083	$-1.47*10^{-2}$	0.051
Temp*E/S	$-0.90*10^{-2}$	0.260	$-0.90*10^{-2}$	0.206
Time*preT	$0.03*10^{-2}$	0.967		
Time*E/S	$-0.90*10^{-2}$	0.260	$-0.90*10^{-2}$	0.206
preT*E/S	$2.14*10^{-2}$	0.015	$2.14*10^{-2}$	0.006
pH^2	$2.74*10^{-2}$	0.001	$2.76*10^{-2}$	0.000
$Temp^2$	$4.85*10^{-2}$	0.000	$4.86*10^{-2}$	0.000
$Time^2$	$-0.34*10^{-2}$	0.682		
$preT^2$	$0.21*10^{-2}$	0.803		
E/S^2	$-0.24*10^{-2}$	0.773		
Other statistics				
R^2	0.822		0.816	
Sum of squares		df [c]		df
residual	$6.43*10^{-2}$	23	$6.63*10^{-2}$	30
pure error	$0.11*10^{-2}$	5	$0.11*10^{-2}$	5
lack of fit	$6.32*10^{-2}$	18	$6.52*10^{-2}$	25
F-value lack of fit	16		12	

[a] Pre-treatment temperature of the whey protein.
[b] Enzyme to substrate ratio.
[c] Degrees of freedom.

E/S is more important, as is reflected by the higher absolute regression coefficient. For pH and temperature the quadratic effects were highly significant, which means that for pH and temperature optimal process settings can be defined. Concerning E/S and hydrolysis time, no maximum is found within the experimental area. The effect of pH and temperature is illustrated in the response surface plot (Figure 1), where the parameters for the other three variables are set on constant values of 2 hours, 45°C and 1% for hydrolysis time, pre-treatment temperature (preT) and E/S, respectively. A minimum IC_{50} value is observed around pH 8 to 9 and hydrolysis temperature of circa 45°C.

Concerning the preT some significant interactive effects were observed (Table 3). The interactive effect between preT and E/S is illustrated by the response surface depicted in Figure 2. At high E/S an increase in preT results in an increase in IC_{50} value, whereas at low enzyme concentrations an increase in preT results in a decrease of IC_{50} value. As was mentioned before, the E/S should be high if a low IC_{50} value is desired, therefore, the preT

should be low. From the above presented results, it can be concluded that optimal ACE-inhibiting activity will be reached at high enzyme concentration, long hydrolysis time, pH 8 to 9 and hydrolysis temperature of circa 45°C. The pre-treatment temperature should be rather low.

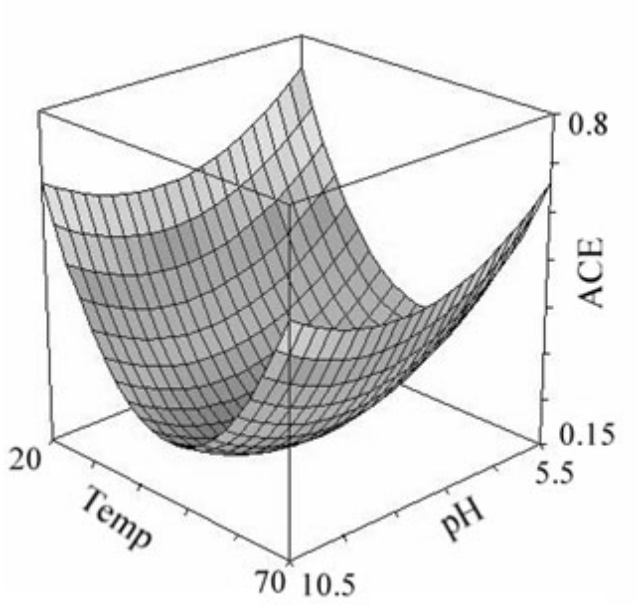

Figure 1: Response surface of the effect of pH and temperature (Temp) on ACE-inhibiting activity expressed as IC_{50} value. The other process parameters were set at their centre value, *i.e.* pre-treatment temperature 65°C, E/S 1% and time 2 hours.

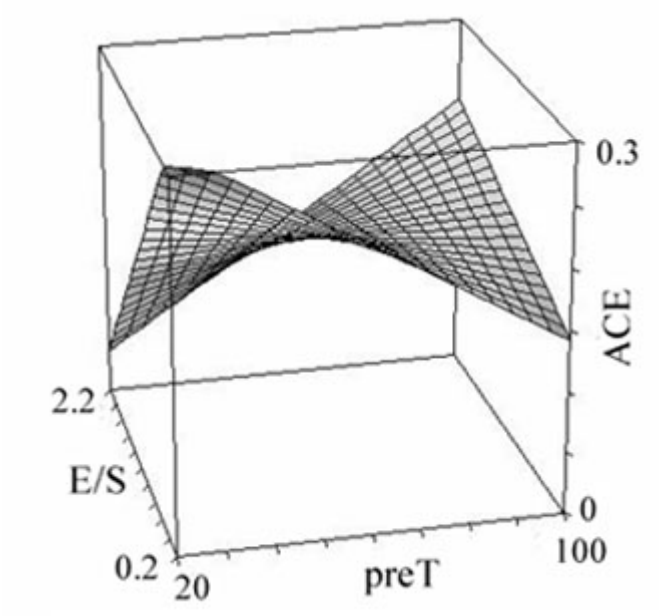

Figure 2: Response surface of the effect of pre-treatment temperature (preT) and enzyme to substrate ratio (E/S) on ACE-inhibiting activity expressed as IC_{50} value. The other parameters were set at their centre value, *i.e.* pH 7.75, temperature 45°C and time 2 hours.

To test the model, new whey hydrolysate samples were produced at optimal process conditions, the pH was set at pH 8 and the pre-treatment and hydrolysis temperature at 45°C. The E/S was set at the maximum value included in the model, *i.e.* 2.19%. The calculated *b*-

coefficients (Table 3) were used to predict IC_{50} values of hydrolysates made at these new process conditions. The predicted IC_{50} value after 2 hours of hydrolysis is 0.091 mg/ml. The inhibition reached with this enzyme concentration (which cannot be increased within the limits of the model) can theoretically be further optimised by increasing the hydrolysis time.
During the hydrolysis at the new process conditions, samples were taken each hour to define the most optimal hydrolysis time. The hydrolysate with the lowest measured IC_{50} value, *i.e.* 0.148 mg/ml, had a DH of 31%, which was reached after 5 hours of hydrolysis (Table 4). The main decrease in IC_{50} value was observed between one and two hours of hydrolysis. Prolonged hydrolysis time resulted only in a slight improvement of ACE-inhibiting activity. At the end of the hydrolysis experiment the IC_{50} value increases again, which implies that there is an optimal hydrolysis time.

Table 4: ACE-inhibiting activity of optimised whey hydrolysates.

Time (hr)	DH	IC_{50} (mg/ml)[a]
1	18	0.202
2	23	0.155
3	27	0.152
4	31	0.155
5	31	0.148
5.5	33	0.196

[a] Concentration whey protein hydrolysate that results in 50% inhibition of ACE.

The ACE-inhibiting activity reached with the optimised hydrolysis conditions did not reach the predicted IC_{50} level. As the enzyme concentration is set at the maximum level, the investigated area is at the edge of the model, which might cause less accurate prediction. The model can be improved in that area by adding new experiments that are performed at high enzyme concentrations. Therefore, we added the optimisation experiments to the model. The extended data resulted, after backward elimination, in a model with 15 regression terms, including quadratic terms for time and E/S (data not shown). The new model predicts an IC_{50} value of 0.148 mg/ml for hydrolysis over 5 hours at the tested optimum conditions. According to the model, it is not possible to decrease the IC_{50} value any further.
The presented results show that response surface modelling can be applied effectively to describe ACE inhibition by protein hydrolysates. Until now, response surface modelling was mostly used to study chemical and physical parameters. Application of this method for optimisation of a bioactive property was not described yet.
Comparison of ACE-inhibiting activity measured in the present study with results obtained by others is precarious, since the IC_{50} values calculated depend on the assay conditions, which varies between the various studies. Moreover, the assay conditions are not always clearly specified. Nevertheless, the attained improvement of ACE-inhibiting activity by process optimisation can be compared to other methods employed to improve ACE-inhibiting activity. The 'centre' settings in the currently used experimental set-up represent average hydrolysis conditions and resulted in an average IC_{50} value 0.19 mg/ml, which could be improved by

23% with the used experimental approach. An alternative method to increase ACE inhibition by hydrolysates that can be applied on industrial scale, is membrane filtration. Effectiveness of this method depends on the parental hydrolysates. Ultrafiltration of tryptic hydrolysates of α-lactalbumin and β-lactoglobulin over 1 kDa membranes resulted in a 2 to 3 fold increase of ACE-inhibiting activity, whereas ultrafiltration of hydrolysates produced by subsequent action of pepsin and trypsin did not increase ACE-inhibiting activity (Pihlanto Leppala et al., 2000). Ultrafiltration of a tryptic hydrolysate of whey protein concentrate over a 3 kDa membrane resulted in an increase of ACE-inhibiting activity by 17%, though subsequent ultrafiltration over a 1 kDa membrane decreased ACE-inhibiting activity again (Mullally et al., 1997). Ultrafiltration of hydrolysates is more expensive than process optimisation, since an extra processing step is introduced. In addition, part of the protein material is lost and the overall amino acid composition might change due to ultrafiltration, which results in decreased nutritional value of the hydrolysate.

ACE inhibition by protein hydrolysates is the result of the inhibiting action of various peptides present. Isolation of one or a few peptides with ACE-inhibiting activity is, therefore, not justified. The method of choice for improvement of ACE inhibition by hydrolysates is the optimisation of the entire peptide composition of hydrolysates, as this is an efficient low cost method, preserving the nutritional value of the protein. Response surface modelling was shown to be an effective method to optimise several parameters of the hydrolysis process simultaneously, resulting in a hydrolysate with maximum ACE inhibition.

AKNOWLEDGEMENTS

The authors thank Rob Verdooren for his support on the statistical analyses.

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Chapter 7

General discussion

INTRODUCTION

Protein hydrolysis is the degradation of proteins by proteolytic enzymes. The combination of selected process conditions and protease specificity controls the peptide composition of the final hydrolysate. Subsequently, the peptide composition determines the molecular and functional properties of the hydrolysate. In fact, modifications in functional properties result from altered molecular propterties (Figure 1). Molecular properties of hydrolysates are commonly charactarised by single factors of the complete hydrolysates, like degree of hydrolysis, surface hydrophobicity or molecular weight distribution. In addition to these 'unifactorial' characterisations, hydrolysates may also be characterised by 'multifactorial' characterisation methods. These multifactorial methods include several molecular properties of the peptides and/or consider the peptide composition rather as a collection of individual peptides than as one group with one common property. In the present study, size exclusion chromatography (SEC), reversed phase chromatography (RPC) and Fourier transform infra red (FTIR) spectroscopy were used as multifactorial characterisation methods. The chromatographic profiles or spectra resulting from these multifactorial characterisation methods can serve as hydrolysate fingerprints. Since these fingerprints are a result of various molecular properties of hydrolysates, they contain information on both molecular and functional properties of hydrolysates (Figure 1).

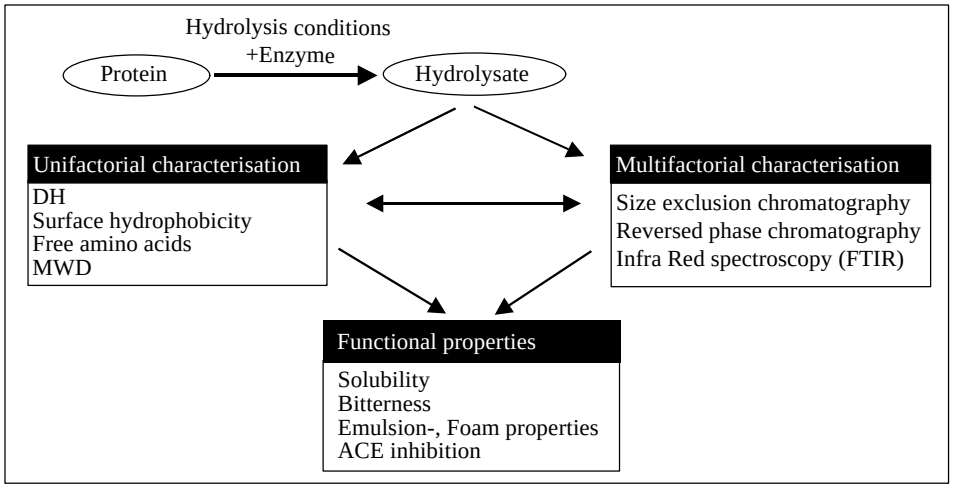

Figure 1: Relations between different hydrolysate characterisation methods.

Research reports concerning protein hydrolysates generally focus either on the molecular or on the functional properties of hydrolysates while only few studies include both aspects (Svenning et al., 1993; Mahmoud, 1994; Caessens et al., 1999d). Most studies on functional properties are limited to a few hydrolysates of which only the DH is measured as biochemical characteristic (Chobert et al., 1988a; Chobert et al., 1988b; Haque & Mozaffar, 1992; Vojdani & Whitaker, 1994; Slattery & Fitzgerald, 1998). Moreover, thorough studies on relationships between different hydrolysate characteristics are very uncommon. Current understanding of biochemical properties of hydrolysates that are important for their functional properties, has been deduced from studies with either fractionated hydrolysates or purified peptides (Lee et al., 1987; Turgeon et al., 1991; Althouse et al., 1995; Huang et al., 1996; Mutilangi et al.,

1996; Caessens et al., 1997; Caessens et al., 1999a; Caessens et al., 1999b; Caessens et al., 1999c; Poon et al., 2001).
Although this reductionistic approach contributes to the knowledge on relations between molecular and functional properties of peptides and peptide mixtures, new developments in computing and analytical sciences creates possibilities for conducting research via a non-targeted approach, starting with non-targeted data-collection. Subsequent post-experimental data processing will reveal new correlations within the data set, which may result in formulation of new hypotheses. Recent developments in *e.g.* proteomics, studying whole protein expression patterns without predefined protein targets, and functional genomics, studying the overall phenotypic change resulting from genetic alterations in stead of focussing on predefined metabolites (Fiehn, 2002), clearly illustrate the benefits of this new holistic approach.

In this thesis, protein hydrolysate characterisation was performed according to such a holistic approach. Hydrolysates were characterised using several multifactorial and unifactorial characterisation methods. Moreover, functional properties of these hydrolysates were determined. Subsequently, research was focussed toward data-analysis (*i.e.* principal component analysis (PCA) and partial least squares (PLS) regression) demonstrating relations between molecular properties and multifactorial characterisation methods, between molecular properties and functional properties, and between multifactorial characterisation methods and functional properties. These correlations were studied for casein hydrolysates and whey protein hydrolysates prepared with several commercially available enzyme preparations.
In addition, the influence of hydrolysis process parameters on hydrolysate properties was determined.

HYDROLYSIS PROCESS CONDITIONS

As depicted in Figure 1, the protease specificity and the process conditions used for hydrolysis determine the final hydrolysate properties. In this thesis, the effect of process conditions on hydrolysate characteristics was studied with regard to the Angiotensin Converting Enzyme (ACE) inhibiting ability of whey protein hydrolysates prepared with a pancreatic enzyme preparation (Chapter 6).
Several process parameters, such as pH, temperature and enzyme concentration, influence the hydrolysis results. The effect of each parameter may also depend on the settings of the other parameters. Therefore, to efficiently optimise the hydrolysis process for maximal ACE-inhibiting ability, experimental design was used. Subsequently, the effects of the process variables (main, quadratic and interaction effects) regarding ACE inhibition were studied with response surface methodology, followed by an estimation of the process conditions that result in a hydrolysate with best attainable ACE-inhibiting activity (Chapter 6). It was shown that process optimisation is not only applicable for regulation of degree of hydrolysis, as was already shown by others (Baek & Cadwallader, 1995; Linder et al., 1995; Diniz & Martin, 1996), but can also be used as a valuable tool for optimisation of functional properties of hydrolysates.

UNIFACTORIAL AND MULTIFACTORIAL CHARACTERISATION METHODS AND THEIR CORRELATIONS

As explained above, hydrolysates can be described by unifactorial as well as by multifactorial characterisation methods. These characterisation methods might reflect similar molecular properties. Therefore, correlations between the profiles obtained from SEC, RPC and FTIR spectroscopy, and the measured unifactorial parameters were determined (Table 1). The correlations are calculated using PLS regression with the chromatographic or spectral profiles as *x*-variables, and the unifactorial variables as *y*-variables.

Table 1: Correlations between unifactorial parameters and multifactorial molecular characterisation methods[a].

	Profiles from multifactorial characterisation					
	Casein			Whey protein		
Unifactorial characterisation	SEC[b]	RPC[b]	FTIR	SEC	RPC	FTIR
DH	++	+++	+++	+	++	++
Free amino acids	±	+++	+++	±	+++	++++
Hydrophobicity (S_0)[c]	±	+++	±	++	++	++
MW fractions:						
>5 kDa	++++	+++	±	++++	++++	++
4-5 kDa	++++	+++	+	++++	+++	±
3-4 kDa	++++	+++	+	++++	++	-
2-3 kDa	+++	±	-	++++	+	-
1-2 kDa	++++	++	+	++++	+++	±
0.5-1 kDa	+++	+++	++	++++	++	++
<0.5 kDa	++++	+++	++	++++	+++	++

[a] Correlation signs are deduced from R^2CV: (-): R^2 <0.5; (±): R^2 0.5-0.7; (+): R^2 0.7-0.8; (++): R^2 0.8-0.9; (+++): R^2 0.9-0.95; (++++): R^2 >0.95.
[b] Correlation based on multivariate analysis with SEC or RPC profiles as *x*-variables.
[c] Measured with ANS as fluorescent probe.

In Table 1, the molecular weight (MW) fractions of hydrolysates are grouped with the unifactorial parameters as with the conversion of SEC profiles into MW fractions relationships between other analytical methods and the molecular weight distribution (MWD) of peptides in hydrolysates can be studied. In terms of unifactorial and multifactorial parameters, MW fractions can be regarded to represent only one single property of a group of peptides, while SEC profiles reflect the separation of peptides on a chromatographic support without any further interpretation thereby considering the contribution of individual peptides. As the MW fractions presented in Table 1 are calculated from the SEC profiles, the correlation between SEC profiles and MW fractions is obvious.

The free amino acid content was low in most hydrolysates except for two enzyme preparations (Flavourzyme and Corolase PP) producing high amounts of free amino acids (Chapter 3). RPC profiles and FTIR spectra predict the amino acid content in hydrolysates adequately. Probably, with these techniques (part of) the individual amino acids can be detected.

In the following, the correlations between two of the unifactorial characteristics, *i.e.* DH and hydrophobicity, and the multifactorial characterisation methods will be discussed in more

detail. Subsequently, the used chromatographic and spectral characterisation methods and their correlations will be discussed.

Unifactorial parameters

Degree of hydrolysis

The degree of hydrolysis characterises hydrolysates in a rather general way and, consequently, hydrolysates with similar DH values may differ in their peptide composition as measured by *e.g.* SEC and RPC (Mullally et al., 1994; Mutilangi et al., 1995; Smyth & Fitzgerald, 1998). Although other hydrolysate properties cannot be deduced from DH, it can be concluded from Table 1 that the DH itself can be predicted from chromatographic analyses or FTIR spectra.

A correlation between SEC profiles and the degree of hydrolysis can be expected since the amount of small peptides will increase with an increase in DH. The DH is also related to RPC profiles and FTIR spectra (Table 1). The correlation with RPC profiles probably exists because peptide molecular weight influences the separation of peptides on RPC (Chapter 2), which implies that the correlation with DH is basically similar to that between SEC and DH. For FTIR spectra, the correlation with DH is probably caused by the change in infrared absorption due to the reduced number of amide bonds after hydrolysis. Amide bonds have a characteristic absorption in the FTIR spectrum (Curley et al., 1998).

A relation between DH and MWD seems obvious and has been reported for hydrolysates prepared with one protein/enzyme combination under various process conditions (Gonzalez-Telllo et al., 1994). In contrast, a study comparing hydrolysates made with different enzyme preparations concluded that MWD and DH were not related (Smyth & Fitzgerald, 1998). However, if one analyses the data presented in the latter report with PLS regression, an outlier is readily identified. This sample appeared to be a hydrolysate prepared with Flavourzyme, which is an enzyme preparation containing considerable exo-peptidase activity (Clemente et al., 1999; Hamada, 2000). Due to the exo-peptidase activity a high DH is reached, while still a significant proportion of large peptides is present. If this sample is excluded from the data set, DH is rather well predicted from the MW fractions ($R^2 = 0.75$). In another study, from one of the same authors (McDonagh & FitzGerald, 1998), it was also concluded that no correlation between MWD and DH exists. In this case application of PLS regression may reveal a correlation between MWD and DH as well.

In conclusion, the degree of hydrolysis is strongly related to MWD and other characterisation methods and provides therefore no additional information on the hydrolysate characteristics.

Hydrophobicity of protein hydrolysates

Hydrophobicity of proteins, especially surface hydrophobicity, are reported to be important for several functional properties such as emulsion- and foam properties (Horiuchi et al., 1978; Kato & Nakai, 1980; Voutsinas et al., 1983; Aluko & Yada, 1993; Jahaniaval et al., 2000). Surface hydrophobicity of proteins may change due to modifications caused by heat treatment and hydrolysis (Bonomi et al., 1988; Mahmoud et al., 1992; Aluko & Yada, 1993; Mutilangi et al., 1995; Wu et al., 1998; Jahaniaval et al., 2000). Extensive protein hydrolysis was reported to cause decreased surface hydrophobicity, which was attributed to the increased net

charge of peptides relative to the parental material (Mahmoud et al., 1992). Protein hydrolysis does not necessarily result in altered surface hydrophobicity, as shown for a rapeseed hydrolysate (Nakai et al., 1980).

Generally, surface hydrophobicity is determined using fluorescence probes like 1-anilinonaphthalene-8-sulfonate (ANS) or cis-parinaric acid (CPA). ANS interacts with aromatic hydrophobic amino acids while CPA interacts with aliphatic hydrophobic amino acids. Hydrophobicity (S_0) is expressed as the increase of the relative fluorescence intensity (RFI) with increased protein concentration (Nakai et al., 1996).

In the present study, the hydrophobicities of hydrolysates and parental proteins were measured with ANS as fluorescent probe. Since protein concentration was measured in mg/ml, S_0 was expressed as ΔRFI/(mg protein equivalent/ml). Hydrophobicity of parental WPC (S_0 = 0.9) was higher than that of casein (S_0 = 0.75). Hydrolysis of casein resulted in decreased hydrophobicities (S_0 < 0.2), which is in accordance with surface hydrophobicity decrease after hydrolysis of casein by Alcalase, α-chymotrypsin and papain as reported by Ludwig et al (1995). The S_0 values of whey protein hydrolysates, having values of 0.04 to 3.4, show that hydrolysis with certain proteolytic enzymes induce increased surface hydrophobicity.

The surface hydrophobicity values of whey hydrolysates appeared to be related to the MWD of hydrolysates (Table 1). With multivariate regression analysis a rather good model for prediction of S_0 was obtained (R^2 = 0.83; S_0 predicted from SEC profiles). For casein hydrolysates, S_0 is correlated with RPC profiles but not with SEC profiles. The differences in hydrophobicities for the casein hydrolysates were small. Possibly, the RPC profiles are better able to explain these small differences due to the better separation of peptides by RPC compared to SEC.

Mahmoud and coworkers suggest that the increase in net charge of peptides relative to the parental protein may overcome the effect of exposed hydrophobic groups (Mahmoud et al., 1992). Smaller peptides will have a higher charge density, which can explain the relation between MWD and surface hydrophobicity found in the present study. Moreover, fluorescent probes have low quantum yields when they are bound to small peptides, even if these peptides are hydrophobic (Nakai et al., 1996). Therefore, it is questionable whether surface hydrophobicity is truly measured using fluorescence probes.

In conclusion, hydrolysate hydrophobicity determined with fluorescent probes depends on other molecular properties of peptides and has, therefore, no value as an characterisation parameter.

Multifactorial characterisation methods

Next to unifactorial parameters, multifactorial chromatographic and spectral profiles of hydrolysates were determined in order to characterise hydrolysates. Separation of hydrolysates on two chromatographic materials, *i.e.* SEC and RPC was studied in this thesis.

Chromatography

The suitability of characterisation of hydrolysates by SEC or RPC was tested by principal component analysis (PCA), which is an unsupervised classification technique (Dillon & Goldstein, 1984). PCA revealed that RPC profiles, in contrast to SEC profiles, discriminate between hydrolysates originating from different protein sources. This is probably the result of a better separation of peptides on RPC when compared to SEC (Chapter 2) as also reported by others (Lemieux & Amiot, 1990; Aubes-Dufau et al., 1995; Madsen et al., 1997).

Although separation of peptides is better on RPC than on SEC, interpretation of RPC profiles is more complicated. Using RPC does not allow for direct indication whether differences found between samples originate from differences in peptide MW or peptide hydrophobicity. On the contrary, chromatograms obtained from SEC can be converted directly to molecular weight fractions by the use of calibration curves. Despite the fact that hydrophobic and electrostatic interactions interfere with the separation on hydrodynamic volume in SEC (Visser et al., 1992; Silvestre et al., 1994; Fujinari & Manes, 1997; Tossavainen et al., 1997), the influence of these interactions on calculation of MWD can be minimised by inclusion of a large diversity of peptides in the calibration curve (Chapter 2).

A clear correlation between RPC profiles and MW fractions of hydrolysates was found (Chapter 2, Table 1) caused by the influence of peptide molecular weight on retention time in RPC (Chapter 2). Due to the better separation, RPC profiles contain more information on the hydrolysate composition and are consequently more discriminative than SEC profiles. In conclusion, RPC is superior over SEC for multifactorial characterisation of hydrolysates.

Infrared spectroscopy

Another multifactorial method used to characterise hydrolysates is infrared spectroscopy (Chapter 5). In complex mixtures many functional groups contribute to the spectra, which impedes interpretation of molecular properties of constituent peptides. However, each spectrum is characteristic for each mixture and can, therefore, be used as a fingerprint.

Principal component analysis showed that FTIR spectra effectively discriminate between hydrolysates made from either whey proteins or caseins and between hydrolysates prepared with acidic enzymes compared to hydrolysates made with either neutral or alkaline enzymes (Chapter 5). These results indicate that FTIR spectra are indeed useful as hydrolysate fingerprints.

As shown in Table 1, the MW fractions and hydrophobicity are stronger related to RPC profiles than to FTIR spectra, which are mainly related to the DH and the free amino acid content of hydrolysates. In conclusion, FTIR spectra are not effectively related to the measured molecular properties of hydrolysates, but are useful as hydrolysate fingerprints.

Practical use of molecular characterisation methods

The correlations between the hydrolysate characterisation methods (Table 1) are summarised in Table 2. The results reveal that reversed phase chromatography is strongly related to all measured unifactorial variables (Table 2). Therefore, it can be concluded that, of the tested methods, RPC is best suited to obtain information on molecular properties of hydrolysates.

Table 2: Correlations between analytical methods.

	Predictors		
Dependant	SEC	RPC	FTIR
DH	moderate	strong	moderate/strong
Free amino acids	weak	strong	strong
Hydrophobicity	weak/strong	strong	weak
MW fractions	strong	strong	weak/moderate

RELATIONS BETWEEN MOLECULAR AND FUNCTIONAL PROPERTIES

The molecular properties of the casein and whey protein hydrolysates, determined with the techniques described before, were studied in relation to several functional properties of the hydrolysates, *i.e.* emulsion- and foam properties, solubility, bitterness and ACE-inhibiting activity.

Emulsion and foam forming

The ability to form emulsions and foams differed between hydrolysates made from caseins or whey protein concentrate (Chapters 3 and 4). Casein hydrolysates formed considerably more foam than the whey hydrolysate samples. This difference in foam-forming ability was also observed for the parental proteins. Other studies mention both whey protein and casein as superior foam-forming proteins (Kuehler & Stine, 1974; Townsend & Nakai, 1983; Lee et al., 1992; Huang et al., 1997; Caessens, 1999; Van Koningsveld, 2001). The use of other foam-forming methods, protein concentrations and/or solvent conditions may explain the different results found.

The ability to form foams and emulsions differed between hydrolysates prepared from different protein sources as well as between hydrolysates of one protein source. Characteristics of emulsion- and foam-forming ability of casein hydrolysates and whey protein hydrolysates are summarised in Table 3. Hydrolysis of whey protein concentrate (WPC) resulted in decreased foam- and emulsion-forming ability relative to intact WPC, whereas a few casein hydrolysates revealed a slight increase in emulsion- and/or foam-forming ability when compared to intact casein.

Directly after homogenisation, several emulsions prepared with casein hydrolysates showed bimodal droplet size distributions with a relatively narrow peak at rather large droplet sizes (high d_{32}, high d_{43}) (Chapter 3). Microscopic examination of these emulsions diluted in SDS showed that the emulsion droplets were actually considerably smaller (approx. < 10 μm), which strongly suggests that the observed large droplets are actually aggregates of smaller emulsion droplets. Otherwise microscopic observations of emulsions having broad droplet size distributions (low d_{32}, high d_{43}), generally revealed the presence of both small and rather large droplets. It seems that in these emulsions aggregation of droplets (partially) resulted in coalescence. In conclusion, it seems that all casein hydrolysates are able to form small emulsion droplets, but that they are not all able to stabilise these droplets.

Table 3: Emulsion and foam-forming properties of casein and whey protein hydrolysates at pH 6.7, (I = 0.075 M).

	Emulsion forming	Foam forming
Whey protein hydrolysates	• Inferior to WPC • Bimodal particle size distributions	• Inferior to WPC • F_0 range: 0-42 ml • F_0 depends on MWD
Casein hydrolysates	• Increased/decreased relative to casein • Emulsions with three types of particle size distributions: – Low d_{32}, low d_{43} – Low d_{32}, high d_{43} – High d_{32}, high d_{43}	• Similar to casein • All hydrolysates high F_0 (F_0 > 40 ml)

Abbreviations: WPC: whey protein concentrate, d_{32}: volume average particle diameter, d_{43}: weight mean diameter, F_0 : initial foam volume of foams prepared by whipping 100 ml of a 0.05% (w/v) protein solution.

Foam-forming experiments showed that all casein hydrolysates form high foam levels (Chapter 4), which is in accordance with the good emulsion-forming ability of these hydrolysates.

Concerning whey hydrolysates, foam-forming ability differed considerably between the samples. The MWD of peptides in whey protein hydrolysates proved to be important for foam-forming ability (Chapter 4). Especially peptides with molecular weights of 3 to 5 kDa appeared to be positively related to high foam levels. It was hypothesised that small whey protein peptides are less likely to be amphiphilic than large whey protein peptides or casein peptides thereby not contributing to foam formation (Chapter 4).

Emulsion-forming ability of whey hydrolysates was inferior to WPC for all tested samples (Chapter 3). Emulsion droplet size distributions seemed similar for all whey hydrolysates. However, if the whey hydrolysate emulsions were examined microscopically after dilution of the emulsions in SDS, distinct emulsion types were observed. Certain contained mainly small emulsion droplets, while others contained only large emulsion droplets (5 to 15 μm) or mixtures of large and small droplets. In contrast to casein hydrolysate emulsions, several whey hydrolysates, *i.e.* the ones forming only large emulsion droplets, exhibit poor emulsion-forming ability.

Comparison of foam- and emulsion-forming ability of the whey protein hydrolysates, reveals that hydrolysates forming relatively voluminous foams (>25 ml) also form small emulsion droplets. However, several hydrolysates that were able to form small emulsion droplets, showed only moderate or low foam-forming ability, and hydrolysates that did not form any foam, did form emulsions, although the emulsion droplets were relatively large. In conclusion, it seems that in case of whey hydrolysates, emulsion formation is more easily attained than foam formation, implying that foam formation requires more stringent molecular properties. During foam formation, instability processes like disproportionation and drainage also occur (Walstra, 1996). Therefore, the difference in foam and emulsion-forming properties may be caused by the inability to oppose foam instability.

Although emulsion formation was not correlated to defined molecular properties, a good correlation between FTIR spectra and emulsion-forming ability was found for both casein and whey protein hydrolysates (Chapter 5). These correlations suggest that factors like peptide conformation and/or amino acid sequence are important for emulsion-forming ability. Emulsion-forming ability of pure peptides from myoglobulin, showed that a peptide containing two α-helices possessed better emulsion-forming ability than peptides containing one of the two helices (Poon et al., 2001). These results suggest that conformation is important in emulsion formation.

Emulsion and foam stability

The foam and emulsion stabilities of the measured whey and casein hydrolysate samples summarised in Table 4, show that hydrolysates behave differently in emulsion and foam stabilisation. The emulsion-stabilising ability of hydrolysates is superior to the foam-stabilising ability, since hydrolysates that form highly stable foams also form stable emulsions, whereas the inverse does not occur. Casein hydrolysates generally stabilise foams better than whey hydrolysates, while whey hydrolysates are better emulsion stabilisers.
Different mechanisms involved in emulsion and foam stability may explain the differences in stabilising ability of the hydrolysates.

Table 4: Relation between foam and emulsion stability of casein and whey protein hydrolysates[a].

	Foam		
Emulsion	High	Moderate	Low
High	CnNwf06, CnVfp04, CnVfp07 WcCpp09	CnBrm01,CnCl102, CnFlz01, CnPem09,CnPem13,CnNwf10 WcBrm06,WcCPP12,WcPem06, WcPem10,WcPep05	WcAlc06,WcBrm05,WcCl106, WcCl107,WcPep10,WcVfp06, WcVfp07
Moderate		CnCl105, CnCPP 18	CnPep06, CnBrm06 WcAlc23,WcPx611,WcPx617, WcP5807,WcP5815,WcFlz09, WcFlz24, WcNwf07, WcNwf14
Low		CnCPP11, CnPep02	CnAlc14, CnAlc19, CnP5815, CnP5822, CnFlz15, CnPx608, CnPx618

[a] For details on sample codes: see Chapter 2.

Concerning emulsions, the stability towards coalescence was monitored. Stability against coalescence is established through steric or electrostatic repulsion between emulsion droplets, or by the ability to oppose the flow of liquid from the film between two approaching droplets by forming interfacial tension gradients. In creamed emulsions, repulsive forces will be the main mechanism concerning prevention of film break, since the droplets are in relative quiescent conditions (Walstra, 1986). From the emulsion stability measurements it appeared that the relatively close packing of emulsion droplets in the cream layer did not result in increased coalescence (Chapter 3). Therfore, it may be concluded that peptides in these hydrolysates are able to generate sufficient steric and/or electrostatic repulsion to prevent too close proximity of the droplets.

In foams, drainage and disproportionation are the most important instability factors (Damodaran, 1997). Disproportionation was only observed for some casein hydrolysate foams. In most foams, drainage seemed the most important instability factor (Chapter 4). To prevent drainage, peptides at the film interface should interact in order to remain adsorbed and thus have the ability to maintain a γ-gradient and form a stagnant surface opposing the downward flow of liquid (Prins, 1999). For these interactions other molecular properties next to those involved in preventing coalescence of emulsion droplets seem to be involved, which is confirmed by the fact that fractions of hydrolysates of different molecular weight appear to contribute to emulsion stability and foam stability (Chapter 3 and 4).

Based on studies with chromatographic fractions of some hydrolysates, or with molecular weight distributions of one or two complete hydrolysates, it is suggested that the minimum molecular weight of peptides needed for emulsion stability is >2 kDa (Lee et al., 1987; Caessens et al., 1999a), >2.5 kDa (Agboola et al., 1998) or >5 kDa (Chobert et al., 1988a). Low molecular weight peptides, <1 kDa (Lieske & Konrad, 1996) or <10 kDa (Althouse et al., 1995; Mutilangi et al., 1996), were reported to contribute to foam stability. All these studies were only able to estimate the minimum molecular weight needed to establish good functional properties, since limitations are set by the choice of the hydrolysate fractions used.
In the present research, first all data on molecular and functional properties of different hydrolysates were obtained, followed by statistical data analysis of these data simultaneously, thereby preventing pre-selection of peptides based on molecular weight fractions or other properties. Therefore, correlations are investigated using all available information, which allows a better definition of the molecular properties of hydrolysates important for emulsion and foam properties. Using this approach, it was revealed that for stabilisation of emulsions against coalescence a minimum peptide length of 2 kDa is needed (Chapter 3), while for foam stability the lower limit is approximately 7 kDa (Chapter 4).

Solubility

In the previous chapters, solubility and bitterness of hydrolysates were only discussed in relation to FTIR spectra (Chapter 5). Similar to the study on emulsion and foam properties relations between molecular properties and solubility, bitterness and ACE inhibition were studied. The results are briefly discussed in order to define the molecular properties related to these functional properties.
The solubilities of all casein and whey protein hydrolysates general increase with increasing DH. However, the correlation between DH and solubility was poor. Correlations between DH and solubility have been found for hydrolysates prepared with single protein/enzyme combinations (Perea et al., 1993; Mutilangi et al., 1996; Slattery & Fitzgerald, 1998), but not for correlations between DH and solubility in general (Vegarud & Langsrud, 1989; Mullally et al., 1994).
All characterisation methods used in the present study, except DH and solubility, were performed on the soluble fractions of the hydrolysates. The solubilities of the complete hydrolysates appeared to be correlated to the RPC profiles of the soluble fractions. If the

solubility increases, a shift from high to low retention times of peptides on the RPC column is observed. Correlation studies between RPC profiles and MW fractions of hydrolysates (Chapter 2) showed that low molecular weight peptides generally elute faster from the RPC column than high molecular weight peptides. Therefore, the correlation between solubility and RPC may result from a relation between solubility and the amount of low molecular weight peptides. If only molecular weight distribution would explain differences in solubility of hydrolysates SEC profiles should correlate well to solubility, which was actually not observed. Therefore, it is expected that hydrophobic interactions, which also influence peptide separation on RPC, also contribute to differences in solubility. This seems reasonable, since the solubility of hydrophilic peptides is better than that of hydrophobic peptides (Mutilangi et al., 1996).

In conclusion, solubilities of hydrolysates seem to be related to the molecular weight distribution as well as to hydrophobicity/hydrophilicity of peptides in the hydrolysates.

Bitterness

Bitterness of hydrolysates was scored for all casein and whey protein hydrolysates on a five-point scale. Bitterness of 2% casein hydrolysate solutions was higher than of 5% whey protein hydrolysates (Chapter 5).

Although bitterness of hydrolysates is reported to increase with increasing DH, possibly reaching a maximum value (Adler-Nissen & Olsen, 1979; Nielsen, 1997), such a correlation was not found in our study. The correlations between DH and bitterness depended mainly on the protein and enzyme source used. Both increase and decrease of bitterness with DH was observed.

Bitterness was correlated to FTIR spectra, although the correlation was not very strong (Chapter 5). In order to assess bitterness, hydrolysates were neutralised before the samples were tasted. Hydrolysates prepared with acidic enzymes became salty, which possibly interfered with the bitterness perception. If these samples were excluded models with better correlations between predicted (from FTIR spectra) and measured bitterness were obtained (Chapter 5). Predictions improved for correlations between bitterness and MWD and between bitterness and RPC profiles. For whey protein hydrolysates the predictive ability of SEC- and RPC profiles is similar to that of the FTIR spectra, whereas for casein hydrolysates prediction from RPC profiles is rather good, but less effective than from FTIR spectra. The regression coefficients resulting from the multivariate regression analysis between MWD and bitterness of whey protein hydrolysates, revealed that the fractions containing peptides of 0.5 to 5 kDa are positively related to bitterness, with the fraction of 0.5-1 kDa being best related to bitterness.

In conclusion, in contrast to other studies, we found no correlation between DH and bitterness. Concerning whey hydrolysates it appeared that especially low molecular weight peptides exhibit bitterness.

ACE inhibition

Whey protein hydrolysates were generally less effective ACE inhibitors than casein hydrolysates. This is in accordance with the generally higher ACE-inhibiting activity of pure casein peptides when compared to pure whey peptides (Chapter 1, Table 4).

No correlations were found between ACE-inhibiting activity and any of the measured molecular properties for casein hydrolysates, whereas in case of whey protein hydrolysates ACE-inhibiting activity was weakly correlated to the MWD. The 0.5-1 kDa MW fraction was most strongly related to high ACE inhibition. Moreover, a positive correlation between the MW fraction of 3-4 kDa and ACE-inhibiting activity was found. Most of the reported peptides exhibiting ACE-inhibiting activity are low-MW peptides (generally <12 amino acids), confirming the positive correlation found for the low-MW peptide fraction of the whey protein hydrolysates.

Many peptides, differing substantially in their amino acid composition, have been reported to be able to inhibit ACE *in vitro* (Meisel & Schlimme, 1996; Yamamoto, 1997; FitzGerald & Meisel, 1999; Clare & Swaisgood, 2000). The fact that numerous different peptides are able to cause ACE inhibition might explain the poor correlations existing between ACE-inhibiting activity and molecular properties.

Summary of correlations between molecular properties and functional properties

The results of the studied correlations between molecular and functional properties of hydrolysates are summarised in Table 5.

Solubilities of total hydrolysates and bitterness of the soluble fractions of hydrolysates prepared with alkaline and neutral enzymes are related to the RPC profiles, which reflect differences in molecular weight distribution and hydrophilicity/hydrophobicity of peptides. In case of whey protein hydrolysates, the correlation between RPC profiles and bitterness is probably caused by differences in MWD, since a rather good relation between MWD and bitterness was found. Therefore, whey hydrolysate bitterness is only tabled in relation to the MW fractions (Table 5).

Table 5: Relation between molecular and functional properties of hydrolysates.

	Molecular property	
Functional property	MW[a] fraction	MWD[a] + hydrophilicity/ hydrophobicity (RPC)
Solubility	—	x
Bitterness[b]	0.5-5 kDa[c]	x[d]
ACE inhibition	0.5-1kDa, 3-4 kDa[c]	
Foam forming	3-15 kDa[c]	
Emulsion forming	—	—
Foam stability	> 7 kDa[d]	x[c]
Emulsion stability	>2 kDa	

[a] MW: molecular weight, MWD: MW distribution.
[b] Relation based on hydrolysates produced with alkaline and neutral enzymes.
[c] Relation applicable for whey protein hydrolysates.
[d] Relation applicable for casein hydrolysates.

ACE-inhibiting activity and foam-forming ability of whey protein hydrolysates were both related to the MWD of the hydrolysates, whereas for casein hydrolysates no relations with molecular properties were found for these functional properties. Emulsion stability of all hydrolysates and foam stability of casein hydrolysates are each correlated to specific molecular weight ranges of hydrolysates. For foam stability considerably larger peptides are needed than for emulsion stability, which suggests that other instability processes are involved in foam- and emulsion stabilisation. Foam stability was correlated to RPC profiles but not to MWD calculated from SEC. This correlation may (partially) be caused by differences in MWD, but cannot be used to deduce a correlation between specific MW fractions and foam stability. Finally, emulsion-forming ability was not related to any of the measured molecular properties of the hydrolysates.

Elucidation of peptide sequences related to functional properties

As discussed before several functional properties are clearly related to the MWD of hydrolysates. These properties are, therefore, determined by peptide length rather than by the amino acid composition of the peptides in the hydrolysates. Some properties like ACE-inhibiting activity and bitterness of casein hydrolysates, and emulsion-forming ability of all hydrolysates could not be related to MWD. For these properties it would be interesting to identify the specific peptides involved and to subsequently elucidate the amino acid sequences in order to, possibly, unravel the molecular properties that are essential for the functionality.

A method to elucidate peptide compositions of hydrolysates is separation of peptides on a RPC column and followed by analysis with mass spectrometry (LC/MS/MS) analysis, Fragmentation of peptides yields mass spectra that may be used to elucidate amino acid sequences of peptides. Computational programs have been developed that combine the masses of the fragments with the amino acid sequences of the original proteins in order to calculate the amino acid sequences of the peptides.

In the present study, the capability of the LC/MS/MS technique to characterise peptide compositions of hydrolysates was tested using a tryptic casein hydrolysate analysed with a LC/MS/MS system as described in Chapter 2. It appeared that only a part of the peptides expected from the tryptic casein hydrolysis were detected. From the primary sequences of the four caseins present in sodium caseinate, only 68, 59, 28 and 22% of respectively α_{s1}, α_{s2}, β and κ-casein, were identified. The missing sequences were either di-and tripeptides or high molecular weight peptides. Relatively short peptides have been reported (Stone et al., 1998) to be poorly identified with the SEQUEST Search program (developed by Yates et al., 1995) used in the present study. Possibly, poor quality of the zoom- and fragmentation spectra prohibited identification of the larger peptides. The inability to identify amino acid sequences of certain peptides by SEQUEST Search was also reported by Stone and coworkers (Stone et al., 1998). They identified only 5 out of 18 unknown peptides.

As a comparison, only a part of the peptides in tryptic digests of other proteins and of peptide fractions of cheese were identified by LC/MS/MS (Mock et al., 1993; Alli et al., 1998; Stone et al., 1998; Spahr et al., 2001). Analysis of tryptic digests of pure proteins resulted in identification of 75% of the amino acid sequence of the human growth hormone (Mock et al.,

1993), 93% of the sequence of β-casein (Tan et al., 1993) and 90% of the sequences of ovalbumin and BSA (Opiteck & Jorgenson, 1997). In the latter study digests were separated over a two-dimensional coupled LC/LC system, which improved the separation of peptides significantly.

In conclusion, determination of the total peptide composition of hydrolysates with LC/MS seems to be too ambitious, especially since peptide compositions of protein hydrolysates prepared with broad activity proteases will be more complex than tryptic digests.

For identification of peptides that are responsible for specific functional properties of hydrolysates not all hydrolysate peptides need to be known. In fact, if peptides that contribute to a specific functional property can be appointed, only the amino acid compositions of these peptides have to be elucidated. A possible approach to deal with this problem is to combine RP-HPLC/MS with multivariate regression analysis. The principal idea is to separate hydrolysates on RP-HPLC and to use the RPC profiles for regression analysis with a functional property of interest as *y*-variable. In this way parts of the RPC profiles that contribute positively to the specific functional property can be identified. Subsequently, the amino acid composition of peptides eluting from the RP column at the retention times of interest can be identified with the data obtained from the MS analysis.

To test the applicability of this method, regression analysis was performed with RPC profiles of casein hydrolysates as *x*-variables and a functional property that was not related to MWD, as *y*-variable. Bitterness, emulsion-forming ability and ACE-inhibiting activity were regarded as *y*-variables. Finally, a set of 21 casein hydrolysates was selected, as these samples resulted in a model with a fairly good correlation between predicted (from the RPC profiles) and measured bitterness (R^2 = 0.81). As the RPC profiles were not correlated to ACE-inhibiting activity and emulsion-forming ability of casein hydrolysates, only the correlation between bitterness and peptide sequences was considered.

Regression coefficients of the model predicting bitterness from the RPC profiles reflect the contribution of retention times in the reversed phase profiles to the bitterness of hydrolysates. With the MS data, amino acid sequences of peptides eluting at retention times with highest positive correlation coefficients with bitterness, were identified from various samples (Table 6).

Certain peptides appear in hydrolysates prepared with different enzyme preparations, whereas other peptides are only found in hydrolysates of one specific enzyme preparation (Table 6). Bitterness of these hydrolysates may differ considerably. The peptide GPFPIIV, for example, is found in hydrolysates with high bitterness, but also in Corolase PP hydrolysates, which demonstrate relatively low bitterness. This peptide has been reported as one of the bitter peptides of casein hydrolysates (Kanehisa et al., 1984; Swaisgood, 1992; Bumberger & Belitz, 1993). The peptide FVAPFPQVFGKEK is found in all three hydrolysates of Newlase F, which have markedly different bitterness intensities. The peptide has high homology to peptides with reported bitterness (FFVAPFPQVFGK, FVAPFPEVF, VAPFPEVF) (Bumberger & Wieser, 1995). Differences in bitterness between the hydrolysates might be caused by differences in the concentration of possibly bitter peptides, or by masking of the bitter taste by other peptides present.

Table 6: Amino acid sequence of peptides eluting from RP column at retention times positively correlated to bitterness and the bitterness scores for the total hydrolysates.

Peptides of α_{s1}-casein			Peptides of β-casein		
Peptide (part of sequence, MW)	Sample	Bitterness score[a]	Peptide (part of sequence, MW)	Sample	Bitterness score[a]
VAPFPQVF	CnAlc14	3.1	HLPLPLLQ	CnAlc14	3.1
(f 25-32, 905.5)	CnAlc19	3.1	(f 134-141, 930.6)	CnPx608	2.7
	CnPep06	3.4	LHLPLPLLQS	CnNwf06	1.7
	CnPep08	3.4	(f 133-142, 1131.4)	CnNwf08	2.5
	CnP5809	3.3		CnNwf10	3.9
	CnCl103	3.5	LPLPLLQ	CnBrm03	3.1
	CnCl102	3.0	(f 135-141, 793.5)	CnBrm06	3.2
	CnPx608	2.7	PPLTQ	CnCl102	3.0
PFPQVF	CnAlc14	3.1	(f 75-79, 555.3)	CnCl103	3.5
(f 27-32, 735.3)	CnAlc19	3.1	GPFPIIV	CnCl102	3.0
	CnP5809	3.3	(f 203-209, 742.5)	CnCl103	3.5
FVAPFPQVFGKEK	CnNwf06	1.7		CnNwf10	3.9
(f 24-36, 1494.8)	CnNwf08	2.5		CnBrm03	3.1
	CnNwf10	3.9		CnBrm06	3.2
				CnP5809	3.3
				CnAlc19	3.1
				CnCpp11	1.6
				CnCpp18	1.3
				CnCpp23	1.4

[a] Bitterness was assessed on a five point scale, a score of five representing extreme bitterness (for details see Chapter 5).

It is therefore likely that the quantity of specific peptides is important for bitterness. Since MS results can not be related to peptide quantities, UV absorption should be used. Therefore, separation should be improved, resulting in single peptide peaks.

In analysis of protein mixtures, two-dimensional (2D) gel electrophoresis has been applied to determine the composition of complex protein mixtures. Although important progress has been attained on 2D gel electrophoresis protocols, detection of different proteins and protein quantification as well as reproducibility, still remains difficult (Gorg et al., 2000). Problems with separation of peptides are even more pronounced because many peptides have similar molecular weights and isoelectric points. Besides separation, fixation of peptides in gels will cause major problems as well.

In protein research, two-dimensional HPLC separation systems have been reported as alternatives for 2D gel electrophoresis. Good results were obtained with separation of proteins on ion-exchange or isoelectro-focussing columns followed by reversed phase chromatography (Wagner et al., 2000; Wall et al., 2000; Chong et al., 2001). These combined systems have also been proven to be useful for separation of peptides from tryptic hydrolysates of pure proteins (Takahashi et al., 1985; Opiteck & Jorgenson, 1997) and for separation of peptides from tryptic digests of complex protein mixtures (Link et al., 1999; Davis et al., 2001). For separation of peptides, capillary electrophoresis may be an important tool, since it has been reported to give good separation of peptides and the separation is based on size and charge which is complementary to the separation RP-HPLC (Madsen et al., 1997; Issaq et al., 1999).

None of these studies, however, reported complete elucidation of the peptide composition of hydrolysates.
These 2D systems will also improve separation of peptides present in hydrolysates prepared from mixtures of proteins with broad specificity proteases. However, separation of peptides in these complex mixtures is hampered by the high similarity between peptides and it will be a major challenge to identify the entire peptide composition of hydrolysates.

Prediction of functional properties using analytical characterisation methods

In the present study several functional properties of hydrolysates were regarded, *i.e.* solubility, bitterness, ACE-inhibiting activity, and emulsion- and foam properties. In order to determine whether analytical characterisation methods can predict functional properties, regression analysis studies with analytical characterisation methods were performed (Chapters 3,4,5). The resulting correlations are summarised in Table 7.
The most generally used method to characterise hydrolysates is the degree of hydrolysis (DH). As stated before in Chapter 1, DH does not provide much information about the hydrolysate properties. In the present research it was shown that DH was not indicative for foam-and emulsion properties of hydrolysates (Chapters 3,4) nor for properties like solubility and bitterness. Molecular weight distribution is correlated to emulsion stability of casein and whey protein hydrolysates, to foam stability of casein hydrolysates and to foam-forming ability of whey hydrolysates. A weak correlation of SEC profiles with solubility was found, and in case of whey hydrolysates, with ACE-inhibiting activity and bitterness of hydrolysates prepared with alkaline or neutral enzymes. For several functional properties, the correlations with SEC profiles are better than with MW fractions, which can be explained by the fact that part of the information is lost when SEC profiles are summarised in MW fractions.

Table 7: Correlation between analytical characterisations and functional properties[a].

	Analytical characterisation method									
	Casein					Whey protein				
Functional property	DH	MWD[b] frac.	SEC[c] prof.	RPC	FTIR	DH	MWD frac.	SEC prof.	RPC	FTIR
Solubility	±	-	+	++	+	-	±	+	+++	++
Bitterness	-	-	- (±)[d]	- (+)[d]	± (+)[d]	-	- (±)[d]	- (+)[d]	- (++)[d]	± (+)[d]
ACE	-	-	-	-	-	-	±	+	+	±
Emulsion										
Forming	-	-	-	-	++	-	-	-	-	++
Stability	-	++	+++	++	++	±	++	++	++	++
Foam										
Forming[e]	-	-	-	-	-	±	+	++	+	++
Stability[e]	-	++	++	++	++	-	-	-	++	±

[a] Correlation signs are deduced from R^2CV: (-): R^2 <0.5; (±): R^2 0.5-0.7; (+): R^2 0.7-0.8; (++): R^2 0.8-0.9; (+++): R^2 0.9-0.95; (++++): R^2 >0.95.
[b] Correlation based on MW fractions.
[c] Correlation based on SEC profiles.
[d] Correlation based on hydrolysates prepared with alkaline and neutral enzymes.
[e] Correlation with MW fractions calculated with results from TSK column (Chapter 4).

The before-mentioned functional properties are also correlated to RPC profiles, which are generally better correlated with the functional properties than the SEC profiles. In addition, RPC profiles account for differences in foam stability of whey protein hydrolysates. Moreover, if only casein and whey hydrolysates prepared with alkaline and neutral enzymes are included, RPC profiles are also correlated to bitterness.
Finally, FTIR spectra were shown to have good predictive abilities for several hydrolysate properties (Chapter 5). Partly, this can be explained by the fact that the FTIR spectra contain information about the degree of hydrolysis and molecular weight distribution. However, FTIR spectra also account for difference in functional properties that were not related to other analytical methods.
The above-described relations between analytical and functional properties of hydrolysates are schematically represented in Figure 2. The figure illustrates that as the analytical characterisation methods account for more molecular aspects of the peptides in hydrolysates, the differences in functional properties are better explained.

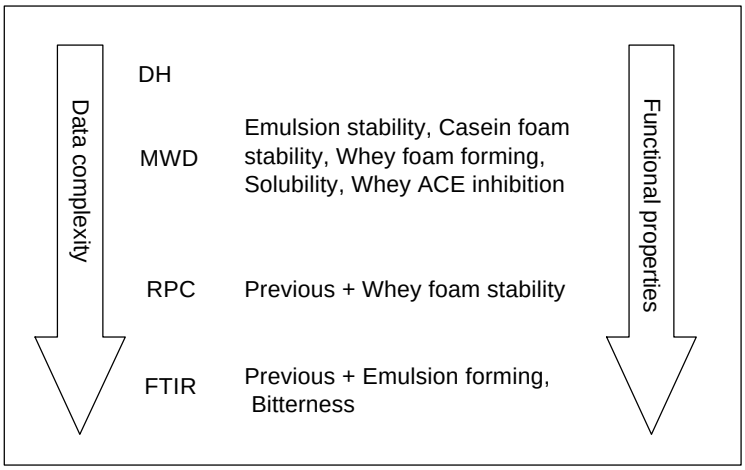

Figure 2: Relation between analytical characterisation methods and functional properties of hydrolysates.

From the applied analytical methods it can be concluded that FTIR spectroscopy is most suited for characterisation of hydrolysates with the aim to use a single analysis for prediction of functional properties of hydrolysates.

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SUMMARY

Proteins are important constituents of the human diet, as they are a source of essential amino acids and nitrogen. For application of proteins in several food products it is necessary to hydrolyse proteins first, for example for use in hypoallergenic infant formula or in clinical nutrition. Protein hydrolysis can be performed with a range of different proteolytic enzymes under various process conditions, which determine the final peptide composition. The peptides resulting from protein hydrolysis differ from the parental material in various molecular properties like molecular weight and charge. These altered molecular properties causes changes in techno-functional properties of hydrolysates like solubility, bitterness, emulsifying and foaming properties. Moreover, apart from the nutritional change (*e.g.* improved digestibility), hydrolysis may result in liberation of bioactive peptides.

The aim of this thesis was to study the molecular and functional properties of casein and whey protein hydrolysates prepared with commercially available, crude, food-grade enzyme preparations, which correspond to hydrolysates used in practice for food products. In order to elucidate the correlations between molecular and functional properties, hydrolysates were characterised in a standardised manner and the data were analysed using multivariate data analysis. Moreover, the ability to use single analytical characterisation methods as hydrolysate fingerprints was investigated.

A general introduction on milk proteins, protein hydrolysis, characterisation of hydrolysates and functional properties of hydrolysates is given in Chapter 1.

In Chapter 2, two different hydrolysate characterisation methods, *i.e.* size exclusion- (SEC) and reversed phase chromatography (RPC) were compared. The RPC profiles appeared to discriminate better between hydrolysates prepared from different protein sources than did the SEC profiles. Moreover, from regression analysis, it appeared that the molecular weight distributions (MWD) of hydrolysates highly were highly correlated to the retention times of peptides on RPC. Finally, for small peptides (< 15 residues) it was shown that retention times on RPC depend on their amino acid compositions. Retention times of peptides obtained from hydrolysates of a specific protein source can be predicted accurately using amino acid retention time coefficients calculated from retention times of peptides that originate from the same protein source.

In Chapter 3, the emulsion properties of 22 whey protein- and 22 casein hydrolysates are studied, showing clear differences in emulsion-forming behaviour of hydrolysates from either protein source. Casein hydrolysates generally showed better emulsion-forming ability compared to whey hydrolysates, whereas the emulsion-stabilising properties of several casein hydrolysates were inferior to that of the whey hydrolysates. Emulsion stability was correlated to the MWD of the hydrolysates. Especially peptides >2 kDa contributed to stabilisation of emulsions.

For the same 44 hydrolysates, foam properties were determined (Chapter 4). Casein hydrolysates appeared to posses good foam-forming ability, though whey hydrolysates generally showed inferior foam forming ability relative to the parental protein. The foam-forming abilities of the whey hydrolysates were related to their MWD. The regression

coefficients from the model that predict foam-forming ability from molecular weight distributions showed that the MW fraction with peptides of 3 to 5 kDa was most strongly related to high foam-forming ability. Concerning casein hydrolysates, foam stability was correlated to the MW of peptides, especially to peptides >7 kDa. Whey hydrolysates were generally not able to form stable foams.

In Chapter 5, it was investigated whether Fourier transform infrared (FTIR) spectra of hydrolysates can serve as fingerprints of hydrolysates and if these fingerprints can predict functional properties of hydrolysates. Indeed, FTIR spectra effectively discriminate between hydrolysates made from different protein sources and different classes of proteolytic enzymes, indicating that FTIR spectroscopy is a valuable fingerprinting method. Moreover, good correlations between FTIR spectra and a range of functional properties was established, also for properties such as emulsion-forming ability, that are not related to the MWD of hydrolysates. As FTIR spectra are relatively easy to measure, new hydrolysates might be analysed with this technique in order to estimate their functional properties.

Finally, the influence of hydrolysis process conditions on the ACE-inhibiting activity of whey protein hydrolysates was studied (Chapter 6). ACE inhibition is caused by a number of peptides present in hydrolysates and, therefore, optimisation of the total hydrolysate for ACE inhibition is most efficient. Protein hydrolysis depends on several process parameters, of which the effect on the final peptide composition depends on the settings of the other parameters. Therefore, hydrolysis process parameters were optimised simultaneously using experimental design for efficient experimentation and response surface modelling for determination of the optimum process conditions. This approach was shown to result in the definition of hydrolysis conditions that yield a whey hydrolysate with best possible ACE inhibition.

In conclusion, the analytical characterisation methods used during the study showed that RPC reflects several molecular properties of hydrolysates like degree of hydrolysis, MWD, surface hydrophobicity and free amino acid content (Chapter 7). Moreover, correlations between molecular properties and functional properties revealed that many functional properties are related to different molecular weight fractions. For example, higher molecular weight peptides are needed to establish foam stability than for emulsion stability. Low molecular weight peptides of whey hydrolysates are both related to bitterness and ACE inhibiting activity. In addition, it was concluded that among the tested methods, FTIR is the best-suited method for hydrolysate fingerprinting.

SAMENVATTING

Biochemische en functionele karakterisering van caseïne- en wei-eiwit-hydrolysaten en de bestudering van de correlatie tussen deze eigenschappen met behulp van multivariate data analyse.

Eiwitten zijn ketens van aminozuren die aan elkaar gekoppeld zijn door middel van peptide-bindingen. De aminozuren die in de voeding zitten worden, na opname, gebruikt om lichaams-eigen eiwitten aan te maken. Daarnaast zijn de eiwitten belangrijk als stikstofbron voor andere bouwstoffen. De vertering van eiwitten in het maagdarmkanaal is belangrijk voor de opname van de aminozuren. Soms verloopt deze vertering niet goed en is de opname van aminozuren te laag. Om de aminozuuropname te verbeteren zijn producten beschikbaar die gehydrolyseerde (in stukjes geknipte) eiwitten bevatten. Een ander mogelijk probleem bij de consumptie van eiwitten is het optreden van allergische reacties, bijvoorbeeld bij baby's die allergisch zijn voor koemelkeiwitten. Het hydrolyseren van de eiwitten kan zo'n allergische reactie voorkomen.
Voor de hydrolyse van eiwitten kunnen verschillende soorten proteases (enzymen die eiwitten knippen) worden gebruikt. Het resultaat van de hydrolyse wordt bepaald door de specificiteit van het enzym en door de gebruikte procescondities, zoals temperatuur en pH. De gevormde peptiden hebben andere eigenschappen dan de oorspronkelijke eiwitten, zoals een lager molecuulgewicht en meer lading. De moleculaire eigenschappen van de peptiden kunnen bepaald worden aan de hand van diverse biochemische technieken, zoals chromatografische scheidingstechnieken.
De veranderingen in moleculaire eigenschappen leiden tot veranderingen in functionele eigenschappen van de hydrolysaten ten opzichte van de oorspronkelijke eiwitten. De functionele eigenschappen kunnen worden onderverdeeld in biologische en technologische eigenschappen. Biologische eigenschappen zijn de nutritionele waarde en de fysiologische (bio-active) eigenschappen van hydrolysaten. Onder nutritionele waarde valt bijvoorbeeld de verteerbaarheid en de (verminderde) allergeniciteit. Bio-active hydrolysaten zijn bijvoorbeeld hydrolysaten die de bloeddruk verlagen of die het ontstaan van trombose remmen. Technologische eigenschappen hebben betrekking op de toepassing van het hydrolysaat in een voedingsmiddel, zoals bijvoorbeeld de oplosbaarheid en het emulsie- en schuimgedrag van eiwithydrolysaten.
In dit onderzoek werden hydrolysaten van melkeiwitten bestudeerd. In melk komen twee belangrijke groepen eiwitten voor: caseïnes en wei-eiwitten. Deze twee typen eiwitten werden gehydrolyseerd met verschillende commercieel verkrijgbare proteases die gebruikt (kunnen) worden in de levensmiddelenindustrie. Diverse biochemische (bijvoorbeeld de hydrolyse graad, molecuulgewichtsverdeling, vrij aminozuur gehalte) en functionele eigenschappen van de resulterende hydrolysaten werden bepaald. Vervolgens werd bestudeerd welke verbanden er zijn tussen verschillende biochemische eigenschappen onderling en tussen biochemische en functionele eigenschappen. Bovendien werd onderzocht of het mogelijk is om meerdere functionele eigenschappen te kunnen voorspellen aan de hand van een enkele biochemische

karakteriseringsmethode. Om deze relaties te onderzoeken werd gebruik gemaakt van statistische methoden, in het bijzonder van multivariate data analyse technieken.
In dit onderzoek zijn diverse analytische technieken gebruikt om de eiwithydrolysaten te karakteriseren. Een belangrijke gangbare methode is de bepaling van de peptide grootte verdeling, gemeten met gel permeatie chromatography (GPC of SEC). Een andere methode om hydrolysaten te karakteriseren is reversed phase chromatografie (RPC), waarbij zowel molecuulgrootte als hydrophobiciteit van peptiden de scheiding beïnvloeden. Zowel SEC als RPC leveren chromatogrammen op die uniek zijn voor ieder hydrolysaat en die daarom als 'fingerprints' kunnen worden gebruikt. Karakterisering van de hydrolysaten met deze twee technieken werd onderzocht (Hoofdstuk 2). RPC profielen bleken beter geschikt te zijn om hydrolysaten van elkaar te onderscheiden dan SEC profielen. Bovendien blijkt dat de peptide grootte verdeling een grote invloed heeft op de scheiding van peptiden met RPC. De informatie over hydrolysaten die wordt verkregen met GPC en RPC is dus voor een deel hetzelfde. Omdat RPC een meer gedetailleerd beeld geeft van ieder hydrolysaat, is het een betere karakteriserings-techniek dan GPC.
Vervolgens werden twee functionele eigenschappen van hydrolysaten apart bestudeerd, namelijk emulsie- en schuimeigenschappen. Emulsies zijn mengsels van olie en water. Omdat olie en water niet spontaan mengen zijn oppervlakte actieve stoffen, zoals eiwitten of peptiden nodig. Bovendien moeten de oliedruppels die gevormd worden stabiel blijven. Als emulsiedruppels met elkaar in contact komen kunnen ze aggregaten vormen (clusters van emulsiedruppels) of ze kunnen samensmelten tot een grotere emulsiedruppel (coalescentie). Door aggregatie of coalescentie zullen de emulsiedruppels opromen (waarbij een laag samengepakte emulsiedruppels ontstaat die op de waterlaag 'drijft'). Eiwitten of peptiden kunnen bijdragen aan de stabiliteit van emulsies door ervoor te zorgen dat de oliedruppels elkaar niet te dicht naderen. De emulsie-vormende en de emulsiestabiliserende eigenschappen van caseïne-hydrolysaten en van wei-eiwit-hydrolysaten werden in dit onderzoek bestudeerd (Hoofdstuk 3).
De bestudeerde caseïne-hydrolysaten vormden over het algemeen beter emulsies, dat wil zeggen vormden beter kleine oliedruppels, dan de wei-eiwit-hydrolysaten. De gevormde emulsies zijn echter niet altijd stabiel. De meeste emulsies die met wei-eiwit-hydrolysaten werden gemaakt vormden een roomlaag. De oliedruppels die zich in de roomlaag bevonden bleven over het algemeen intact (er trad geen of weinig coalscentie op). De stabiliteit van de emulsies gemaakt met caseïne-hydrolaten varieerde sterk. Sommige emulsies waren even stabiel als emulsies gemaakt met intact caseïne, terwijl andere emulsies snel oproomden of helemaal niet stabiel waren hetgeen resulteerde in de afscheiding van een olielaag. Uit de correlatie tussen de molecuulgewichtsverdeling van de peptiden in de hydrolysaten en de stabiliteit van de emulsies ten opzichte van coalescentie, bleek voor zowel caseïne- als voor wei-eiwit-hydrolysaten te gelden dat peptiden die groter zijn dan 2 kDa belangrijk zijn voor de emulsiestabiliteit.
Alle caseïne-hydrolysaten waren in staat om veel schuim te vormen, terwijl de schuimvorming door wei-eiwit hydrolysaten onderling sterk varieerde (Hoofdstuk 4). De schuimvorming door

wei-eiwit-hydrolysaten was afhankelijk van de molecuulgewichtsverdeling van de peptiden. De fractie met peptiden met een molecuulgewicht van 3-5 kDa bleek het meest bepalend voor de schuimvormende eigenschappen. Net als de emulsiestabiliteit, was ook de stabiliteit van schuim gemaakt met caseïne-hydrolysaten afhankelijk van de molecuulgewichtsverdeling van de peptiden. Peptiden met een molecuulgewicht van minimaal 7 kDa zijn nodig voor het stabiliseren van schuim. Voor schuimstabilisering zijn dus grotere peptiden nodig dan voor het stabiliseren van emulsies.

De karakterisering van de hydrolysaten werd, naast SEC en RPC, uitgebreid met een andere fingerprinting methode, namelijk infrarood (FTIR) spectroscopie (Hoofdstuk 5). Hierbij wordt het infrarood spectrum van de complete hydrolysaten gemeten, waarbij het resultaat wordt bepaald door het totaal van peptiden dat in het mengsel aanwezig is. Met deze techniek kan geen uitspraak gedaan worden over individuele peptiden, maar het levert een unieke spectrum op voor ieder hydrolysaat. Analyse van de FTIR spectra van alle caseïne en wei-eiwit-hydrolysaten met multivariate data analyse liet zien dat de spectra goed onderscheid kunnen maken tussen hydrolysaten gemaakt uit verschillende eiwitbronnen en met verschillende typen enzymen. Vervolgens werd bestudeerd of de FTIR-spectra kunnen worden gebruikt om functionele eigenschappen van hydrolysaten te voorspellen. Eigenschappen die voorspeld werden op basis van de molecuulgewichtsverdeling van peptiden (beschreven in Hoofdstuk 3 en 4) werden ook goed voorspeld met FTIR spectra. Bovendien was het mogelijk om andere functionele eigenschappen zoals de bittere smaak van hydrolysaten, oplosbaarheid en emulsievormende eigenschappen te voorspellen. Deze FTIR spectra zijn relatief eenvoudig te meten en kunnen dus worden gebruikt om een inschatting te maken van de functionele eigenschappen van nieuwe hydrolysaten.

Tot slot werd onderzocht of de ACE remmende activiteit van wei-eiwit-hydrolysaten, gemaakt met één enzympreparaat, kan worden gestuurd door de hydrolysecondities aan te passen (Hoofdstuk 6). Het 'Angiotensin Converting Enzyme' (ACE) is een enzym dat op diverse plaatsen in het lichaam voorkomt en is betrokken is bij de regulatie van de bloeddruk. Remming van dit enzym zou een bloeddruk verlagende werking kunnen hebben. Veel verschillende peptiden zijn *in vitro* in staat om dit enzym te remmen. Hydrolysaten zijn een verzameling van peptiden en voor maximale ACE remming zouden zoveel mogelijk ACE remmende peptiden in het hydrolysaat aanwezig moeten zijn. Zodra een combinatie van eiwit en protease is gekozen, wordt de peptidensamenstelling van een hydrolysaat bepaald door de procescondities. De invloed van procesparameters op de hydrolyse is afhankelijk van de instelling van de andere parameters. Om tot goede optimale condities te komen werden de instellingen van de procescondities gekozen volgens 'experimental design', een statistische methode die erop gericht is om met zo min mogelijk experimenten een proces zo goed mogelijk te bestuderen. Vervolgens werden de resultaten van de verschillende procescondities geanalyseerd met lineaire regressie analyse (response surface modelling), waaruit de procescondities die een hydrolysaat met maximale ACE

remming opleveren werden bepaald. Het bleek dat de ACE remming van de wei-eiwit-hydrolysaten goed gestuurd kon worden.
Als afsluiting worden in Hoofdstuk 7 de gevonden resultaten bediscussieerd. Uit vergelijking van de geteste analytische methoden blijkt dat scheiding van hydrolysaten met RPC profielen oplevert die informatie bevatten over diverse moleculaire eigenschappen van hydrolysaten zoals de mate van hydrolyse, de molecuulgewichtsverdeling van hydrolysaten, oppervlakte-hydrophobiciteit en gehalte aan vrije aminozuren. Betreffende de relaties tussen moleculaire en functionele eigenschappen is gebleken dat de molecuulgewichtsverdeling gerelateerd is aan diverse eigenschappen zoals emulsiestabiliteit van zowel caseïne- als wei-eiwit-hydrolysaten, aan schuimvorming, bitterheid en ACE-remming van wei-eiwit-hydrolysaten en aan schuimstabiliteit van caseïne-hydrolysaten. Deze eigenschappen zijn ook gerelateerd aan RPC profielen, die in het algemeen een betere voorspelling van de functionele eigenschappen oplevert. Tot slot is karakterisering met FTIR spectroscopie de beste manier om 'fingerprints' van hydrolysaten te maken en kunnen diverse functionele eigenschappen worden voorspeld aan de hand van de FTIR spectra.

NAWOORD

Vier jaar aan één onderwerp werken leek me van tevoren bijna onmogelijk. Maar het is me toch gelukt. Dankzij de combinatie van het promotieonderzoek en het Numico-onderzoek heb ik een leuke afwisseling gehad tussen de druk van het bedrijf en de diepgang van wetenschappelijk onderzoek, met aan het einde natuurlijk de druk van het schrijven van het boekje. Ik wil hier iedereen bedanken die me geholpen heeft om dit allemaal mogelijk te maken.

Op het moment dat ik bij Numico begon, was de researchafdeling net verhuisd naar een aantal barakken in Wageningen. De labtafels moesten eerst grondig schoongemaakt worden voordat we konden beginnen, maar al snel begon het echte werk. Dankzij de begeleiding van Hanno Cappon, die de eerste anderhalf jaar projectleider was, was er vanaf het begin een duidelijke structuur in het onderzoek. Hanno, bedankt voor je ondersteuning en interesse in die periode van het onderzoek en de hulp bij mijn eerste kennismaking met multivariate date-analyse en experimental design. Jouw inzichten om het hydrolyseonderzoek op een andere manier vorm te geven dan gebruikelijk zijn een belangrijke peiler geworden van dit proefschrift. Samen met Elmo Wissing heb je me ondersteund bij het vinden van een promotor en het vinden van een goede balans tussen enerzijds het product en proces gericht onderzoek en anderzijds het promotieonderzoek. Elmo, bedankt voor de interesse in het onderzoek en de tweewekelijkse discussies, die met name een steun waren toen Hanno een andere functie binnen Numico kreeg. Hoewel je in de loop van die tijd minder betrokken was bij het project, kon ik altijd even binnenlopen voor een vraag. Dries, jij nam de begeleiding van Hanno over en hoewel het project toen al aardig op stoom was ben je nauw betrokken geweest bij het promotiedeel van het onderzoek. Je kritische opmerkingen bij de manuscripten waren zeer nuttig. Hoewel ons overleg niet erg structureel was had ik altijd het gevoel dat ik met vragen bij je terecht kon waarmee je me vaak direct kon helpen. Je bijdrage aan de grote lijn van het onderzoek was zeer prettig.

Na een jaar onderzoek was het inmiddels duidelijker waar de mogelijkheden voor een promotie-onderzoek lagen. Fons Voragen, ik ben erg blij dat je bereid was om als promotor het onderzoek te ondersteunen. Hoewel de onderzoeksmethode afwijkt van de andere onderzoeken binnen de vakgroep stonden jij en Harry Gruppen daar positief tegenover. De input van jou en Harry heeft voor een belangrijk deel bijgedragen aan de inhoud en de voortgang van het promotieonderzoek. De voortgangsbesprekingen waren altijd heel constructief. Harry, jij hebt het meeste bijgedragen aan de totstandkoming van de manuscripten. Het was erg prettig dat je je eerst op de grote lijn richtte en daaraan op de details. Je hebt de vaart er goed ingehouden wat geresulteerd heeft in het afronden van de promotie in bijna vier jaar. Ik heb de samenwerking altijd erg prettig gevonden. Ook Sander en Gerrit van de vakgroep levensmiddelenchemie, bedankt voor jullie hulp bij praktische werkzaamheden en alle andere vragen.

Ook bij Numico heb ik van diverse mensen ondersteuning gehad bij het onderzoek. Gerrit de Vrij, bedankt voor alle analyses die je voor me hebt uitgevoerd. Dankzij je jarenlange ervaring kon ik altijd vertrouwen op de resultaten en ondanks de drukke bezetting van de HPLC kon er

altijd wel een plekje worden gevonden om mijn monsters te analyseren. Ton en Miew-Woen, jullie hebben vele hydrolysaten gemaakt en geanalyseerd, bedankt voor jullie bijdrage in het project. Gerda, ook jij hebt in het begin in de binnenhaven nog even aan het hydrolysatenproject gewerkt. Ook alle stagiaires wil ik hier bedanken. Yvonne, Kalok, Yvonne, Willem, Arthur, Diane en Koen, bedankt voor jullie inspanningen en ook al is jullie bijdrage misschien niet direct terug te vinden in het proefschrift, het heeft het hydrolysatenproject zeker vooruit geholpen. Ton van Baalen en Bas van der Does, bedankt voor het uitvoeren en ondersteunen van grote schaal hydrolyses en filtraties. Ewald, Wilbert, Marcel en Lizet, er waren de nodige raakvlakken tussen de projecten waarin we werkten en goed overleg bleek toch wel erg nuttig! Tot slot, Rob Verdooren, bedankt voor de statistische ondersteuning van het onderzoek. Bij twijfel kon ik altijd met mijn vragen bij je terecht. Dankzij je adviezen was het mogelijk om mijn resultaten eens op een andere manier te bekijken.

Een deel van het project werd uitgevoerd in samenwerking met het ATO, hoewel dat werk hoofdzakelijk los stond van mijn promotieonderzoek. Karin en Johan, bedankt voor jullie medewerking en interesse in de voortgang van mijn promotietraject. Sorel, your contribution on Chapter 5 was indispensable. Apart from the practical FTIR work your contribution on the manuscript and on the data-analysis was very worthwhile.

Naast iedereen die aan het project heeft gewerkt wil ik hier alle collega's van Numico bedanken voor de gezellige tijd en prettige samenwerking. Gerda, je bent bijna tegelijk met mij bij Numico begonnen en we zijn in die jaren veel met elkaar opgetrokken. Bedankt voor de gezellige lunches en je hulp op het lab. Klaske, ik heb erg veel plezier gehad bij je op de kamer. Bedankt voor de interesse in mijn onderzoek en de frisse kijk die je als 'buitenstaander' erop kon geven. Ik vind het erg leuk dat je mijn paranimf wilt zijn. Verder alle afdelingsgenoten van Biom en van NID, evenals andere Numicoters, bedankt voor de gezellige uitjes, lunches, koffiepauzes, enzovoorts!

Naast het werk moet er natuurlijk tijd zijn voor ontspanning. Gelukkig zijn er voldoende mensen die me daar regelmatig aan herinneren. Richard en Sylvia, Johan en Jolande, bedankt voor alle leuke activiteiten die jullie de laatste jaren hebben georganiseerd. Hopelijk heb ik binnenkort weer de tijd om jullie eens voor te zijn. Brechtje, Sonja, Marjanka, Marjon, Diana, Corien en Femke, ik vind het altijd heel gezellig met z'n allen. Brechtje, ik vind het leuk dat je straks mijn paranimf wilt zijn! Basketballers, met zo'n gezellig team is het onmogelijk om met basketbal te stoppen.

Pappa en mama, bedankt voor jullie interesse en vertrouwen in mij. Het is prettig dat ik met alle vragen altijd bij jullie terecht kan.

Ten slotte Wobbe, na mijn eindspurt van de laatste maanden, hebben we nu weer meer tijd om bijzondere en leuke dingen te gaan doen en ons huis eindelijk echt af te gaan maken!

CURRICULUM VITAE

Cornelly van der Ven werd op 20 juli 1973 geboren te Zeist en groeide op in Driebergen. In 1991 behaalde zij haar VWO diploma aan de Katholieke Scholengemeenschap 'de Breul' te Zeist, waarna zij voor een jaar naar Frankrijk vertrok. In Montpellier werd een jaar later het diplôme d'études françaises aan L'institut des étudiants étrangers, Université Paul Valéry Montpellier III, behaald. In 1992 begon zij haar studie Levensmiddelen-technologie aan de toenmalige Landbouw Universiteit Wageningen, waarin ze zich specialiseerde in de richting biotechnologie. Als onderdeel van haar studie deed zij zowel een 5-maands afstudeervak bij de vakgroep Proceskunde als bij de vakgroep Industriële Microbiologie. Tevens liep ze 5 maanden stage bij het Centre de Recherche STELA, Université Laval, Quebec, Canada. Tot slot liep ze drie maanden stage bij R&D Biotechnologie, AVEBE, Foxhol. In 1997 studeerde ze af met lof.
Na haar studie werkte ze twee maanden bij TNO-voeding in Zeist, waarna ze in 1998 bij Numico Research, Wageningen ging werken. Van 1998 tot 2002 deed ze daar onderzoek naar eiwithydrolysaten. In 1999 werd de leerstoelgroep Levensmiddelenchemie van de Wageningen Universiteit betrokken bij het project om vorm te geven aan het promotie-onderzoek, dat heeft geleid tot het resultaten die staan beschreven in dit proefschrift.
Sinds april 2002 is zij werkzaam bij de business unit Food & Food Processing van ATO B.V. te Wageningen.

Printing: Ponsen & Looyen bv, Wageningen

The research described in this thesis was performed at and financed by Numico-Research, Wageningen