Introducing enzyme selectivity as a quantitative parameter to describe the effects of substrate concentration on protein hydrolysis

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Thesis

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Abstract

To understand the differences in peptide composition that result from variations in the conditions of enzymatic hydrolysis of proteins (e.g. substrate concentration) the mechanism of hydrolysis needs to be understood in detail. Therefore, methods and tools were developed to characterize and quantify the peptides formed during enzymatic protein hydrolysis. The information obtained was used to introduce a novel quantitative parameter: the selectivity of the enzyme towards the individual cleavage sites in the substrate, within the given specificity of the enzyme applied. The selectivity describes the rate of hydrolysis of a cleavage site compared to the rate of hydrolysis of all cleavage sites in the parental protein. Large differences in the selectivity of the enzyme towards the cleavage sites after the same type of amino acid residues in a protein were found. For β-lactoglobulin hydrolyzed by *Bacillus licheniformis* protease the selectivity was found to vary between 0.003 % and 17 % or even 0 for some cleavage sites. The effects of increasing substrate concentration and pH on the hydrolysis were studied. An increase in substrate concentration results in lower kinetics of hydrolysis, related to the available amount of water. This also resulted in significant changes in the enzyme selectivity towards the cleavage sites for which the enzyme has a high selectivity. Changing the pH of hydrolysis resulted in large changes in the kinetics of hydrolysis as well as in the enzyme selectivity. Due to the detailed analysis of the peptide composition, certain a-specific peptides were identified. It was shown that these originate from spontaneous cleavage of formed peptides. The changes in the mechanism of hydrolysis were compared to simulation data. The simulation data were obtained from a stochastic model based on random selection of the substrate and the cleavage site, given the specificity of the enzyme. A quite good agreement was obtained between simulated and experimental data. The parameters and methods developed in this study to describe the mechanism of hydrolysis can potentially be used for more complex systems.

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Chapter 1

General Introduction

Enzymatic protein hydrolysates are used as ingredients in food industry for their techno-functional and nutritional properties, which differ from the properties of intact proteins. The aim of this thesis was to describe the changes in the hydrolysis process and hydrolysates as a function of initial protein concentration. This PhD thesis project was part of an EU project entitled 'Leangreenfood' which had as objectives to reduce the use of chemicals, water and energy in the production of food ingredients by using enzymes at increased substrate concentrations. It is generally observed that increasing substrate concentration results in a slower hydrolysis. It soon became apparent that new methods and terminology needed to be developed to describe the enzymatic hydrolysis process and the hydrolysates. While hydrolysates are commonly used, little is known on the precise enzyme action. Also, there is a lack of methods to describe the kinetics and the sequence of events in enzymatic hydrolysis process on the molecular level.

Characterization of the hydrolysis

The enzymatic hydrolysis of proteins and the resulting hydrolysates are commonly characterized by five descriptors. (**figure 1**) However, as will become apparent, often only a few of these descriptors are used, resulting in partial characterization, rather than in a complete overview. (**table 1**) The descriptors and the way they are used will be discussed in more detail in the next sections.

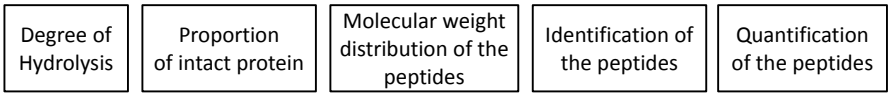

Figure 1. Characterization descriptors of the enzymatic hydrolysis process and hydrolysates.

Degree of hydrolysis

Enzymatic protein hydrolysis is usually monitored by following the degree of hydrolysis (DH), which is defined as the percentage of hydrolyzed peptide bonds over the total number of peptide bonds present. The DH can be determined by several methods; e.g. spectroscopic methods on samples taken at different time points or real time monitoring using a pH-stat device. The increase in the number of free primary amino acids is determined resulting from the reaction of reagents with the free primary amino acids (e.g. colorimetric or formol titration). Upon real-time monitoring with a pH-stat device, use is being made of the protonation/dissociation equilibria of the free amino group or free carboxyl group. These depend on the pH set and the DH is directly estimated from the amount of NaOH or HCl added to keep the pH constant during enzymatic hydrolysis [1]. Common reagents used for spectrophotometric determination of the DH are ninhydrin [2], *o*-phtaldialdehyde (OPA) [3], or trinitrobenzene sulphonic acid (TNBS) [4]. From the DH, the determination of the average peptide chain length (PCL) is sometimes used as an additional calculation to estimate the extent of degradation [5]. The PCL is calculated as PCL=100/DH(%) [6].

Table 1. Overview of studies on enzymatic protein hydrolysis and hydrolysates.

Substrate	Enzyme	Variables studied	DH	Remaining intact protein	Mw distribution	Kinetics parameters[a]	Peptide release kinetics	Reference
β-Casein	Trypsin	One condition	-	-	-	-	Following 9 peptides as a function of time	[7]
Whey protein concentrate	Alcalase, Trypsin, Pepsin	pH and temperature	-	+	-	-	Following 10 peptide peak areas as a function of time	[8]
Whey protein concentrate	Alcalase 0.6, Protease 660, PEM 2500	Enzyme and substrate concentrations (2 to 80 $g.L^{-1}$)	+	-	Chromatographic area versus DH	-	-	[9]
β-Lactoglobulin, α-lactalbumin	Alcalase, Neutrase, PEM, PTN	4 Enzymes on both substrates	+	-	Average molecular mass and average chain length	-	-	[10]
Whey protein isolate	BLP	One condition	-	-	-	-	Identification of 30 peptides at DH = 6.8 %	[11]
β-Lactoglobulin	Trypsin	Temperature	+	-	-	-	Identification of 5 peptides at DH = 1 % and 15 at DH 5 %	[12]
Whey protein isolate	Cardosin A	Enzyme concentration and E/S ratio	-	+	-	+	-	[13]
β-Lactoglobulin, α-lactalbumin	Cardosin A, Cardosin B	Type of enzyme	-	+	-	-	Identification of 35 peptides at one time point	[14]
Whey protein concentrate	Alcalase 0.6, Protease 660, PEM 2500	Enzyme and substrate concentrations (40 to 130 g/L)	+	-	-	+	-	[15]
Whey protein concentrate	Alcalase 0.6, Protease 660, PEM 2500	Enzyme and substrate concentrations (40 and 110 g/L)	+	-	-	+	-	[16]
Whey protein concentrate	Trypsin, Pepsin	Whey from different origins (ovine, caprine, bovine)	-	+	Peak area by Mw range	-	-	[17]
Whey protein concentrate, caseins	BLP	pH and temperature	-	+	-	+	-	[18]
α_{S1} Casein, β-casein	Bovine and camel chymosin	Type of enzyme	-	+	-	-	25 Peptides followed as a function of time	[19]
β-Lactoglobulin	Bromelain, Papain, Pepsin, Trypsin, Endoproteinase Arg-C, CarboxypeptidaseY	Type of enzyme	-	+	-	-	Peak area of 5 RP-HPLC peaks as a function of time	[20]

+/-. descriptor studied/not studied

[a] Kinetics parameters, such as K_M and V_{max} from Michaelis-Menten model

This value is, however, only an approximation and does not provide information on the molecular weight (Mw) distribution. The DH is used as an indication of the extent of the degradation. However, two hydrolysates with similar DH may have very different peptide profiles, resulting from differences in the mechanism of hydrolysis. For such information other descriptors are needed.

Proportion of intact protein

In the Linderstrøm-Lang theory on protein hydrolysis, two models were distinguished: zipper and one-by-one [21]. The objective of these two models is to identify different mechanisms of hydrolysis based on the accessibility of the intact protein to the enzyme. In the one-by-one model, the hydrolysis of the intact protein is slow compared to the hydrolysis of formed intermediate peptides. Consequently, proteins are broken down 'one-by-one', resulting in slower decay of remaining intact protein as a function of DH, as compared to the hydrolysis by the 'zipper' mechanism. (**figure 2**) In the zipper mechanism, described for denatured proteins, the hydrolysis of the intact protein is fast and leads to a lot of intermediate peptides [21].

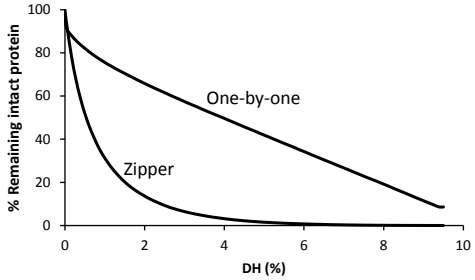

Figure 2. Example of the proportion of remaining intact protein as a function of the DH for the one-by-one and zipper model.

The degradation of intact haemoglobin was, for instance, qualified as one-by-one. However, a zipper mechanism was observed when the haemoglobin was denatured in the presence of urea during hydrolysis [22]. The proportion of remaining intact protein is usually determined by liquid chromatography, by following the peak area, either by reversed-phase high performance liquid chromatography (RP-HPLC) [23] or by size exclusion chromatography (SEC) [17].

Molecular weight distribution

At similar DH and similar proportion of remaining intact protein, peptide profiles can be different. To distinguish such samples, the molecular weight distribution of the peptides can be determined. This is commonly done by SEC analysis of samples taken at different time points or DH values during the hydrolysis [24]. The distribution is typically based on division of the total chromatogram (with UV detection) into separate regions, corresponding to certain molecular weight ranges. (**figure 3**)

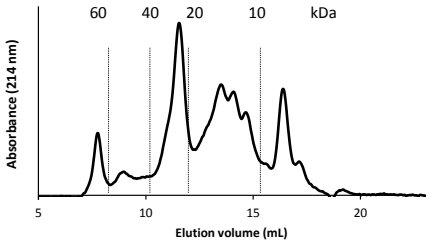

Figure 3. Size exclusion chromatogram of 1 % (w/v) whey protein hydrolysate (ran on Superdex 75 column with 10 mM sodium phosphate buffer pH 8.0 in the conditions described in chapter 2).

This approach is useful in the case of complex protein mixtures to obtain generic information on the degradation and peptide profiles. There are already 153 possible peptides ((N+1)(N+2)/2) resulting from the hydrolysis of β-lactoglobulin, hydrolyzed by an enzyme that only hydrolyzes after glutamic acid residues (N=16). In addition, SEC analyses have been used to correlate the composition of hydrolysates to their techno-functional properties. For instance, it has been described that to obtain a stable emulsion, the solution should have a relative high proportion of peptides with molecular masses larger than 2 kDa [25]. For other purposes (i.e. biofunctional properties, or study of the hydrolysis mechanism) the molecular weight distribution is not sufficient and the different peptides need to be identified and quantified.

Identification of peptides

For identification of peptides mass spectrometry techniques, such as UHPLC-MS/MS or MALDI-TOF-MS, are used. With UHPLC-MS/MS the peptides are separated online by LC before MS detection. With MALDI-TOF-MS, the hydrolysates are either applied directly or first separated into fractions, after which all peptides in each fraction are analyzed simultaneously [26]. MALDI-TOF-MS is, for instance, used in proteomics studies for identification of parental proteins based on the identification of peptides [27]. To identify the parental protein, the MS and MS/MS spectra obtained are matched against databases [28]. In this type of studies, the quality of the annotation is determined by the amino acid sequence coverage with sequence coverages reaching typically only 50-65 % [29]. This parameter is only an indication of the number of unique annotated amino acids over the total number of amino acids in the protein sequence [30]. While such coverage values of 50-65 % are considered sufficient to identify the parental protein, they are not sufficient for a complete quantitative description of the hydrolysate in mechanistic studies. Therefore, LC-MS is preferred, since it will result in on-line separation of the peptides before MS detection for complete analysis of the hydrolysate. This is necessary as hydrolysates are complex mixtures that can contain hundreds of different peptides, as stated above. When LC-MS is applied in protein hydrolysis studies, identification of peptides is often aimed at identifying a small number of peptides to study the influence of hydrolysis conditions on the formation of certain peptides. (**table 2**) Other studies aimed to understand the hydrolysis kinetics based on

the peptides but did not annotate all peptides or did not consider if all peptides had been annotated. (**table 2**) For instance, a study on tryptic hydrolysis of β-lactoglobulin only identified 20 peptides and reached an amino sequence coverage of 85 % [31].

Table 2. Overview of studies on peptide annotation which aimed at understanding hydrolysis.

Substrate	Enzyme	Peptides annotated	Aim	Function of time/DH	Quantification	Reference
β-Lactoglobulin	BLP	23	Aggregating peptides	At one DH	No	[32]
β-Lactoglobulin	Alcalase	130	Gelation	At one time point (gelation)	No	[33]
β-Casein	Trypsin	10	Kinetics of peptide	Time	Arbitrary units (UV_{214})	[7]
α-Lactalbumin	BLP	18	Aggregation	Two time points (60 and 200 min)	No	[34]
β-Casein	$PrPt_1$ of *Lactococcus lactis*	15	Peptide formation modeling	Time	Yes (M)[a]	[35]
β-Casein	BLP	25	Influence of temperature	5 Time points	No	[36]
α_{S1} Casein, β-casein	Chymosin	80	Influence of hydrolysis conditions	Time	Yes (M)[b]	[19]
β-Lactoglobulin	Trypsin	20	Time dependent release kinetics	Time	Yes (M)[a]	[31]
α_{S2} Casein	Trypsin	42	Time dependent release kinetics	Time	UV peak area 215 nm	[37]
α_{S1} ; α_{S2} and β- Casein	Pancreatin	52	Identification of phosphopeptides	DH	MS peak area extracted ion	[38]
β-Casein	Pepsin	41	Study of hydrolysate composition	One time point (3h)	No	[39]
β-Lactoglobulin	BLP	28	Aggregation	One DH	Yes (M)[a]	[40]

[a] Studies that used UHPLC-UV_{214} for exact quantification of the peptides in molar concentration.
[b] Calculation of the molar concentration of the peptides but without considering the UV cell geometry and constant.

Quantification of the peptides

Most studies that aim to quantify peptides focus on a small number of peptides and use quantification based on mass spectrometry signals. However, to achieve a correct quantification, isotopically labeled peptides are needed as reference to correct for differences in ionization [41]. Since labeled variants of all peptides in an hydrolysate would be too expensive, typically only a few references are used. As alternative to the quantification using labeled peptides, semi-quantification based on the changes in MS intensity of the peptides has been used. In the study of hydrolysates, this technique

does not allow comparison of the peptides to each other within a single hydrolysate due to differences in ionization of the peptides [38]. The other disadvantage of such techniques is of course that they do not provide absolute quantification, in terms of molar concentration, of each peptide.
As alternative to the MS based quantification, a quantitative technique has been developed to calculate the exact molar concentrations of all peptides based on the UV_{214} signal of the peptides [35]. In this method, the MS detection is used to identify the peptide, after which the molar extinction coefficient is calculated based on the amino acid composition and number of peptide bonds [42]. Consequently, there is no need for labeled peptides. This method was used for instance to quantify the peptides obtained at one DH [40] or for a large number of peptides as a function of time [35]. (**table 2**)

Applications of protein hydrolysates

As stated above, enzymatic protein hydrolysates are used in the food industry because of their techno-functional properties. For example, solubility and emulsifying properties are different from the properties of intact proteins [43,44]. In detail, the solubility of soy glycinin hydrolysates is higher than the solubility of the intact protein at pH values close to the pI of the protein ($4 < pH < 6$). The solubility of intact glycinin is, however, higher than the one of the hydrolysates at $pH > 7$ [45]. The emulsifying capacities of whey protein hydrolysates obtained by trypsin treatment were improved compared to the emulsifying capacities of the intact proteins. To obtain good emulsifying properties the peptides should not have a molecular mass lower than 5 kDa on average [46]. The hydrolysates have a lower foaming capacity than the intact proteins except at DH lower than 1 % [47]. For this type of application, typically only the DH and the molecular mass distribution of the hydrolysates are considered.
Enzymatic protein hydrolysates are also used to increase the nutritional quality of food products, such as infant formula, weight control products or sport nutrition [48]. The hydrolysates have a better digestibility, faster absorption and a lower allergenicity than the parental protein [49]. Moreover, certain specific peptides formed during enzymatic hydrolysis may have additional bio-functional properties, such as mineral binding, antioxidant, potentially anti-carcinogenic or immunomodulatory properties [50,51]. Due to a variation in conditions of hydrolysis or in the degree of hydrolysis, different hydrolysates are obtained with different amounts of specific peptides. This results in differences in the (bio-)functionality of the total hydrolysate. For instance, the ACE-inhibitory activity of collagen hydrolysates increases with the DH up to DH 4 %. Further hydrolysis (e.g. up to 15 % DH) did not result in a further increase of ACE-inhibitory activity [52]. Another example is found in the successive hydrolysis of bovine whey proteins by pepsin at different pH values followed by a pancreatic enzyme. Pepsin hydrolysis at pH 4 led to a higher residual antigenic activity of the hydrolysates than at pH 2 or 3 [53]. These studies focused on the total hydrolysates while many others tried to identify the peptides responsible for the activity by first fractionating the hydrolysate and isolating the active peptides e.g. [54].

Kinetics of hydrolysis

Enzyme activity

To understand the mechanism of protein hydrolysis and the formation of specific peptides, proteases need to be described in terms of their activity towards the substrate. The activity of enzymes in general and proteases in particular, defines the rate at which the substrate is converted, or at which the intermediate products are formed, and subsequently converted. In protease assays, the enzyme activity is determined based on the formation of product or degradation of the substrate after a set amount of time. With this, the activity of the protease is calculated at a defined pH and a defined temperature. Examples of common assays to determine protease activity are the azocasein assay [55] and the TNBS assay [4].

Rate of hydrolysis

Enzymatic protein hydrolysis is, as mentioned above, described by the DH, which is monitored as a function of time. To characterize and compare the DH vs. time curves obtained, kinetic parameters are used such as the maximum rate of hydrolysis (V_{max}) and the constant K_m described by Michaelis-Menten model. K_m is the concentration of substrate [S] at which the rate of hydrolysis v is half of its maximum as described by equation (1).

(1) $$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

The overall hydrolysis rate determined on the DH curves is fitted to this equation. While it is used, the underlying assumptions do not agree with the system. Consequently it is also found that the fits are not good [56]. The Michaelis-Menten model was developed to describe the conversion of a single substrate to a single product. The kinetic parameters are determined by plotting the so-called Michaelis-Menten plot which describes the initial rate of enzymatic reaction as a function of substrate concentration at constant enzyme concentration [57]. In enzymatic protein hydrolysis there are different cleavage sites within one protein. In addition, the intermediate peptides formed are also substrates for the enzyme. As a result, the enzymatic hydrolysis process starts from a single protein as substrate and leads to the formation of a large number of peptides, which are substrate themselves to the enzyme for further hydrolysis. (**figure 4**) Hence, the standard Michaelis-Menten model cannot be used for enzymatic protein hydrolysis due to the multitude of substrates [58]. In addition, the degree of hydrolysis used to describe the number of bonds cleaved does not reflect the rate of substrate conversion as described by Michaelis-Menten model.

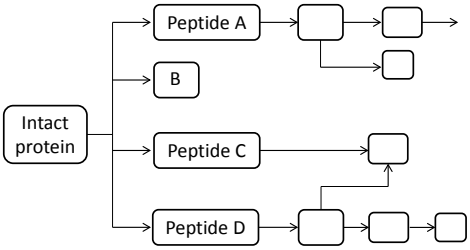

Figure 4. Sequence of events in enzymatic protein hydrolysis [35].

While most of the studies focus on the DH to describe protease kinetics, some have monitored the degradation of intact protein to describe the hydrolysis kinetics. For example, the kinetics of hydrolysis of β-casein was studied by following the molar concentration of the intact protein as a function of time [59]. The kinetics was best fitted by a competitive inhibition model. Other studies have described the peptide release kinetics, which refers to the formation and breakdown of peptides during the hydrolysis process [31,37].

Factors that influence the hydrolysis

Substrate concentration

In enzymatic protein hydrolysis at constant enzyme concentration, it is expected that the same number of bonds as function of time is cleaved independently of the substrate concentration. Based on this, the DH reached at comparable time of hydrolysis is expected to decrease proportionally to the increase in substrate concentration. A deviation from this linearity has been observed for a large diversity of substrates by comparing the DH reached [60,61]. This decrease in rate of hydrolysis with increasing substrate concentration at constant enzyme concentration was explained by competitive product inhibition occurring during enzymatic protein hydrolysis [62]. Because the phenomenon has been observed for different substrates and enzymes, it seems that it is due to a generic property of the system. If the enzyme to substrate ratio is maintained constant (by increasing the enzyme concentration proportionally to the increase in substrate concentration) an increase in the total number of bonds cleaved is expected with increasing substrate concentrations, leading to a rate of hydrolysis and degree of hydrolysis independent of the substrate concentration. It was, however, shown that higher concentrations lead to lower degrees of hydrolysis than expected [15]. In addition to decrease kinetics, increasing the substrate concentration also changes the molecular weight distribution. For instance, at 1 % (w/v) casein, less accumulation of intermediate peptides is observed than at 0.25 % (w/v) casein [63].

The influence of substrate concentration on the kinetics has often been reported, but no satisfactory explanation was found to explain the generic effect of increasing concentration. In the case of proteins the first suspected changes are in the state of the protein or in its aggregation. β-Lactoglobulin is mainly found as a dimer in the pH range

3.5-7.5/8 [64-66]. An increase in the ratio dimer/monomer of β-lactoglobulin is observed with increasing concentration up to 100 $g \cdot L^{-1}$, at 0.1 M NaCl and pH 6.9 [67]. Also, structures larger than dimers seem to be present at concentrations above 100 $g \cdot L^{-1}$, as determined by small angle neutron scattering (SANS) [67]. Besides oligomerization and aggregation, other parameters have been considered when increasing the protein concentration, such as the viscosity of the solutions and the stability of the protein. The viscosity increases linearly up to 25 % (w/w) whey protein concentration, and reaches a value of 25 mPa.s at this concentration [68,69]. Above 25 % (w/w) the correlation between viscosity and protein concentration is not linear due to an increase in molecular interactions between the proteins in the solution [69]. With respect to the stability of proteins when increasing protein concentration, it was shown that the denaturation temperature of pure β-lactoglobulin increases from 67 to 69 °C by increasing the concentration from 10 to 100 $g \cdot L^{-1}$. This indicates a limited effect of protein concentration on its stability [70].

In addition to the state of the protein, it has been suggested that a reversed or transpeptidation reaction can occur during hydrolysis at high substrate concentration [71]. The gel or the water-insoluble products formed by incubation of an hydrolysate present in high concentration with a protease are sometimes referred to as plastein products [72]. Some authors use the term plastein to refer to the product resulting from a condensation or transpeptidation, (i.e. longer peptides) [73]. Others, use the term plastein to describe the aggregation of small peptides by non-covalent bonds [74].

The formation of aggregates and gels during hydrolysis has been observed and studied at high protein concentrations. Solutions of whey proteins isolate (WPI) are described to gel after few hours of hydrolysis with BLP at concentrations of 12 % (w/v) [75]. Gelation of WPI in the presence of Alcalase was also described at concentrations of 20 % (w/v) after 5 hours of hydrolysis [5]. This gel was characterized as plastein product with non-covalent interactions with a comparable chain length before and after gelation. The aggregation or gelation is a property of hydrolysates occurring at high protein concentrations that might be of interest for techno-functional properties.

Conditions of hydrolysis

Besides the changes in substrate concentration, the conformation of the substrate before the hydrolysis is also a parameter that can influence the kinetics of the hydrolysis. For instance, by pre-heating β-lactoglobulin at 80 °C before hydrolysis with Corolase, the time to reach DH 5 % was reduced by a factor 2 [76]. The rate of hydrolysis was also increased by increasing the urea concentration during hydrolysis of β-lactoglobulin by trypsin [77]. Changing the hydrolysis conditions (e.g. pH or temperature) also affects the hydrolysis kinetics or enzyme mechanism. The hydrolysis of whey proteins at increasing temperature (50 to 70 °C) of hydrolysis or at increasing pH (pH 7 to 9) lead to a higher rate of hydrolysis [16]. Furthermore, at elevated temperature of hydrolysis, a decrease in remaining intact protein is found for the hydrolysis of β-lactoglobulin by trypsin after comparable time of hydrolysis [23]. These

examples show the importance of the substrate conformation and of the conditions of hydrolysis on the kinetics of the hydrolysis, and/or on the mechanism of hydrolysis. Complete understanding of the influence of these parameters require a description of the hydrolysates from the degree of hydrolysis to quantitative information on the peptides formed. The focus in the studies of hydrolysates is generally on the influence of one condition on one parameter without aiming at complete overview. (**table 1**) For instance, most studies determine the proportion of remaining intact protein without correlating it to the DH. In the same way, molecular weight distribution studies do not always make a parallel with the proportion of intact protein hydrolyzed. (**table 1**)

Mechanism of hydrolysis

Characterization of proteases

To understand the enzymatic hydrolysis process and the sequence of events, it is first necessary to characterize the protease used. Two types of proteases are distinguished: exoproteases and endoproteases. Exoproteases, also referred to as amino-peptidases or carboxy-peptidases, are described to release terminal amino acids [78]. Endoproteases cleave peptide bonds, which are not on terminal amino acids. Proteases are defined by their specificity, which refers to the type of amino acid after which the enzyme can hydrolyze a peptide bond. The enzyme specificity is used as an indication of the cleavage sites where the enzyme is active. Specific exoproteases cleave the terminal amino acid for only one or two type of amino acids. So, these enzymes do not degrade total protein [79]. Specific endoproteases cleave next to one or two different amino acid residues of the protein leading to the degradation of the parental protein into a diversity of peptides. In this thesis, *Bacillus licheniformis* protease (BLP, E.C. 3.4.21.19) is used. It is a specific glutamyl endoprotease, which cleaves only after glutamic acid and aspartic acid residues, so with Glu or Asp in the P1 position [80]. (**figure 5**)

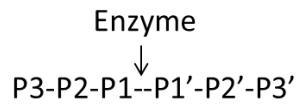

Figure 5. Amino acid denomination at the cleavage site [81].

BLP is a serine protease with 222 amino acids and a molecular mass of 23.6 kDa [82]. The active site of the enzyme is formed by the catalytic triad: His47, Asp96 and Ser167 [83]. In this active site, Ser is the nucleophile that attacks the carbonyl group of the amino acid of the substrate. His is present to stabilize the tetrahedral intermediate formed as the result of the nucleophilic attack and finally Asp stabilises the charge on the His residue. An intermediate acylenzyme complex is formed as the result of this reaction, and the first peptide is released. The acylenzyme complex is subsequently

deacylated after attack by a water molecule, yielding a second tetrahedral intermediate that finally collapses releasing the Ser and the second peptide [84].
In this study, along with the BLP enzyme, the commercial preparation Alcalase is also used. Subtilisin is the main protease found in Alcalase. It is an a-specific protease that cleaves next to aromatic amino acids or acidic amino acids [33]. It is obtained from the same source as BLP.

Preference of the protease
While the enzyme specificity is often mentioned, it is only a partial description of the action of the enzyme. It describes which peptide bonds (after certain amino acids) are cleaved, but does not indicate the rate at which these different peptide bonds (at different locations in the protein sequence) are cleaved. It has, for instance, been observed that some expected cleavage sites, such as Lys-1 and Lys-191 in the hydrolysis of α-$_{s2}$-casein are not cleaved by trypsin [37], as well as 5 different Glu residues in β-casein which were not cleaved by BLP [36]. This indicates that even if the enzyme is specific for a type of amino acid not all expected cleavage sites are used. This property has only rarely been addressed. If done, it is referred to as the preference for certain cleavage site or the affinity of the enzyme towards specific peptide bonds [37]. There are indications that this preference depends on the conditions of the hydrolysis (pH and temperature), but this was never quantified. In addition, this preference may be affected by the amino acids surrounding the cleavage site, also referred to as the 'subsite'. (**figure 5**) In the subsite model, the type of amino acids, but also the charge state of the amino acids on the cleavage site might play a role in the preference of the enzyme towards certain cleavage sites. Still, this preference has never been quantified for real protein substrates. The preference of the enzyme is usually determined by synthetic peptides by calculating a rate of hydrolysis. In this way, it has been established for BLP, for instance, that the presence of a negative charge (Asp) next to the cleavage site (position P1') was poorly accepted by the enzyme [80]. However, the actual preference of the enzyme towards each individual cleavage site in a protein during protein hydrolysis has not been described in a quantitative manner.

Aim and outline

To understand the effects of hydrolysis conditions (e.g. pH, substrate concentration) on enzymatic hydrolysis, a complete description of the kinetics is necessary. In addition, to increase the understanding on the mechanism of enzymatic protein hydrolysis, the kinetics of peptide formation should be described. This can only be done by identification and quantification of all peptides present in the hydrolysates at different time points. Hence, the aim of this thesis is to describe the hydrolysis process, the peptides formed and the mode of action of the enzyme and to develop the required methods to achieve this description.

The hypothesis is that not all cleavage sites in a protein are cleaved with the same rate by the enzyme. Furthermore, we expect that increasing substrate concentration will result in changes in the enzyme preference towards the individual peptide bonds.

In **chapter 2** the effects of substrate concentration on hydrolysis were compared with different enzymes to identify how generic these observations were. To understand the influence and the role of water in the enzymatic hydrolysis process, water activity, free water content (by NMR) and water availability (theoretical) were studied at increasing substrate concentrations (**chapter 3**).
To further describe the influence of system conditions on the hydrolysis, detailed analysis of the hydrolysates is carried out. In **chapter 4** a method was developed to identify the enzyme selectivity (i.e. relative rate of hydrolysis of the cleavage site over the rate towards all cleavage sites in a protein). This method was subsequently applied to study the effects of substrate concentration on the enzyme selectivity (**chapter 5**). The parameters and tools developed to describe the selectivity of the enzyme were also used to study the influence of the pH of hydrolysis (**chapter 6**). During peptide annotation, several a-specific peptides (i.e. peptides non-expected based on the specificity of the enzyme) were observed. The formation of these a-specific peptides is discussed in **chapter 7**. Data obtained from a simulation model for the enzymatic hydrolysis of protein was used to compare to experimental data (**chapter 8**). Finally, in **chapter 9** the challenges in identification and quantification of peptides as well as the developments of the new parameters are discussed.

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Chapter 2

Effects of ionic strength on the enzymatic hydrolysis of diluted and concentrated whey protein isolate

Abstract

To identify the parameters that affect enzymatic hydrolysis at high substrate concentrations, whey protein isolate (1-30 % (w/v)) was hydrolyzed by Alcalase and Neutrase at constant enzyme to substrate ratio. No changes were observed in the solubility and the aggregation state of the proteins. With increasing concentration, both the overall hydrolysis rate and the final DH decreased, from 0.14 to 0.015 s^{-1} and from 24 to 15 %, respectively. The presence of 0.5 M NaCl decreased the overall hydrolysis rate for low concentrations (to 0.018 s^{-1} for 1 % WPI), resulting in similar overall hydrolysis rates for all substrate concentrations. The conductivity increase (by increasing the protein concentration, or by addition of NaCl) has significant effects on the hydrolysis kinetics, but the reason for this is not yet well understood. The results show the importance of conductivity as a factor that influences the kinetics of the hydrolysis, as well as the composition of the hydrolysates.

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Introduction

Enzymatic protein hydrolysis is typically studied at substrate concentrations up to 10 % (w/v). If higher substrate concentrations can be used, the total amount of water and energy used in the process will be decreased. The higher concentrations can, however, affect the protein hydrolysis. Typically, in literature a decrease in the rate of hydrolysis is observed at higher substrate concentrations. As will be discussed in the next section, this is not expected, nor is there a satisfactory explanation for this phenomenon. Therefore, the aim of this work is to identify which factor is the main reason for the decreased rate of hydrolysis at increased substrate concentrations.

The decrease in the rate of hydrolysis at higher substrate concentrations has been shown in several cases: for the hydrolysis of casein (up to 8.5 % (w/v)) [1], for rapeseed protein isolate (up to 13 % (w/v)) [2], for pure bovine hemoglobin (0.1-0.8 % (w/v)) by Alcalase [3] and for hydrolysis of whey protein concentrate (WPC) for three enzyme preparations (MKC Protease, Alcalase, and PEM) [4]. In all cases an increase of the substrate concentration at constant enzyme concentration resulted in a decrease in the rate of hydrolysis (as expressed in the change in DH over time). This decrease in the rate of hydrolysis is also described by equation 1 [5].

(1) $$\frac{dDH}{dt} = k\frac{E}{S_0}e^{-aDH}$$

in which k is the hydrolysis rate constant (s^{-1}), S_0 the initial substrate concentration ($g \cdot L^{-1}$), E the enzyme concentration ($g \cdot L^{-1}$), and a an inactivation parameter. Equation 1 was rewritten into a more general form [4] (equation 2) for determination of the hydrolysis parameters (k and a):

(2) $$DH = \frac{1}{a}\ln(1 + k\frac{E}{S_0}at)$$

From equation 1 it follows that at constant enzyme concentration an increase of the substrate concentration should lead to a proportional decrease in the hydrolysis rate. The number of peptide bonds cut per time unit should be constant with the substrate concentration at constant enzyme concentration (**figure 1A**), but since a higher protein concentration is present, this translates to a lower DH. (**figure 1B**) This, however, is not what is observed in the experimental results. Therefore, it has been suggested that a protease inhibitor might be present in the protein source [4]. Since the observation is repeated for different substrates and different enzymes, it seems that the more than proportional decrease in the rate of hydrolysis with increased substrate concentrations is due to a generic property of the system, rather than due to the presence of a specific inhibitor.

Which property is related to this effect is not known. It has been suggested that the differences in kinetics could be due to the increased viscosity [6]. However, the viscosity of WPI solutions increases only to 25 mPa·s for a 40 % (w/v) solution [7]. During hydrolysis, the viscosity might be further increased, due to aggregation of the

formed fragments. This can eventually lead to gelation during the hydrolysis, which is indeed observed for whey protein isolate hydrolyzed at 20 % (w/v) protein by Alcalase [8].

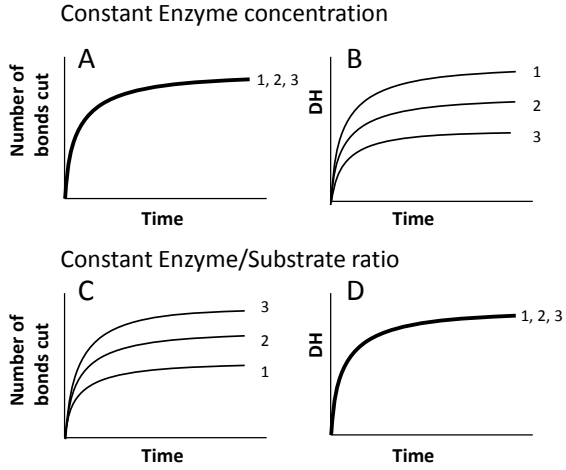

Figure 1. Overview of hydrolysis kinetics for constant enzyme concentration (A, B) and constant enzyme/substrate ratio (C, D), based on equation 1, at low (1), intermediate (2) and high (3) substrate concentration.

To understand which parameter is involved in the decreased hydrolysis kinetics with increasing substrate concentrations, whey protein isolate was hydrolyzed at concentrations ranging from 1-30 % (w/v) at constant enzyme to substrate ratio. Under these conditions, the number of cuts per time unit increases with substrate concentration, while the rate of hydrolysis is independent of the substrate concentration (**figures 1C and 1D**).

The physical properties of the system (e.g. viscosity) were determined as well as the hydrolysis kinetics, the activity toward intact protein, and the peptide profiles.

Materials and Methods

Materials. Bipro, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc. (Le Sueur, MN). The protein composition (by weight) was 74.0 % β-lactoglobulin, 12.5 % α-lactalbumin, 5.5 % bovine serum albumin, and 5.5 % immunoglobulin (from which >75 % IGg1) [9] according to the manufacturer. The protein content of the powder was 93.4 % (w/w) as determined by Dumas (Nx6.32). The nitrogen-to-protein conversion factor (N) for WPI (see **table 1**) was based on the amino acid composition of the proteins as found in Uniprot (www.uniprot.org). Alcalase 2.4L (Subtilisin A from *Bacillus Licheniformis*, batch no PMN 05087) and Neutrase 0.8L (*Bacillus amyloliquefaciens,* batch no PWN 10034) were obtained from Novozymes (Bagsvaerd, Denmark). All other chemicals were of analytical grade and purchased from Sigma or Merck.

Table 1. Properties of whey proteins.

	Uniprot no.	% (w/w) in Bipro	Mw ($g \cdot mol^{-1}$)	# amino acids	N-factor (g of protein /g N)	ε_{280} ($M^{-1} \cdot cm^{-1}$)
β-Lactoglobulin	P02754	74	18281	162	6.34	17210
α-Lactalbumin	P00711	12.5	14186	123	6.24	28460
Bovine serum albumin	P02769	5.5	66432	583	6.08	42925
IgG1	n.a.[a]	5.5	145000[b]	1218[c]	6.49[c]	176900[b]

a. not available.
b. From Tewari & Mukkur [10].
c. Since no sequence was available for IgG1, the number of amino acids was calculated using the average weight of an anhydro amino acid (119 Da) and the reported Mw of the protein, the N content was calculated using the average N content of the other three proteins (1.31 ±0.03 mole N/mole amino acid).

Protein hydrolysis

Sample preparation. Hydrolysis experiments were performed with protein suspensions (in the presence of nondissolved material) or protein solutions (after removal of the nondissolved material). The protein suspensions were prepared by dispersing the WPI powder in Millipore water at concentrations ranging from 1 to 30 % (w/v) and stirring overnight at 4 °C. The solutions were prepared by first dispersing the WPI powder at 45 % (w/v) in Millipore water and stirring overnight at 4 °C. After bringing back the dispersion to room temperature, this dispersion was centrifuged (30 min, 4000 g, 20 °C). The protein content (determined by Dumas), as well as the protein composition (determined by SEC) of the supernatant of the 45 % centrifuged dispersion is similar to that of the pellet. This shows that the precipitation is due to the high amount of proteins, rather than the presence of an insoluble fraction. The protein concentration in the supernatant was determined by measuring the absorbance at 280 nm after diluting 900 times, using a weight-based extinction coefficient of 1.05 $L \cdot g^{-1} \cdot cm^{-1}$. This value ($\varepsilon_{WPI}$) was calculated from equation 3, using the molar extinction coefficient of each protein (ε_i) as found in Uniprot (www.uniprot.org) and the fraction (f_i) of each protein in WPI divided by the molar mass (M_i) of the corresponding protein. (**table 1**)

(3) $$\varepsilon_{WPI} = \sum_{i=0}^{n} \frac{\varepsilon_i \cdot f_i}{M_i}$$

The supernatant was subsequently diluted to the required concentrations (1-30 % (w/v)) in water, in 0.1 M NaCl or in 0.5 M NaCl. In all these conditions, the proteins were soluble (i.e. 94 ± 2.7 % remained in solution after centrifugation). Furthermore, SEC results showed the absence of soluble aggregates (>trimers).

Hydrolysis Conditions. The hydrolysis was performed using a pH-stat. For 1, 5, 10, 20, 30 % (w/v) protein, the pH was kept constant at pH 7 or 8 by addition of 0.2, 1.0, 2.0, 4.0, 6.0 M NaOH, respectively. These concentrations of NaOH were used to keep the added volume constant for the different substrate concentrations. The hydrolysis experiments were performed with Alcalase. For this, 10 mL WPI suspensions or solutions were preheated for 10 min at 40 °C and the pH was adjusted to pH 8 with

NaOH before addition of Alcalase (0.13 µL enzyme/mg protein). To confirm the effect of substrate concentration, as well as the effect of removal of the nondissolved material hydrolysis experiments with Neutrase were performed. For these, the WPI suspensions or solutions were preheated for 10 min at 50 °C and the pH was adjusted to pH 7 with NaOH before addition of Neutrase (0.13 µL enzyme/mg protein). The overall hydrolysis rate was calculated from equation 2, using k_{hydr} as the fitting parameter with $k_{hydr} = k\frac{E_0}{S_0}a \cdot$

The degree of hydrolysis (DH) was calculated from (4)

(4) $$DH(\%) = V_b \cdot N_b \cdot \frac{1}{\alpha} \cdot \frac{1}{m_p} \cdot \frac{1}{h_{tot}} \times 100$$

With V_b the volume of NaOH added in mL; N_b the normality of NaOH; α the average degree of dissociation of the α-NH group (1/α = 1.20 at 40 °C and pH 8 and 1/α = 2.27 at 50 °C and pH 7) [11]; m_p the mass of protein in g; h_{tot} = total number of peptide bonds per gram protein substrate (8.5 mmole/g for whey proteins isolate). The h_{tot} was calculated by multiplying the number of peptide bonds of each protein (B_i) as found in Uniprot (www.uniprot.org) to the fraction of the protein (f_i) in WPI divided by the molar mass (M_i) of each protein (presented in **table 1**) using equation 5.

(5) $$h_{tot(WPI)} = \sum_{i=0}^{n} \frac{B_i \cdot f_i}{M_i}$$

Samples were taken during hydrolysis at various DH (1.5, 3, 4.5, 6, 9 and 12 %). The enzymes were permanently inactivated by adjusting the pH to 2 using 5 M HCl as soon as the samples were taken. Hydrolysates obtained from 20 and 30 % were diluted to 10 % with Millipore water after pH adjustment to avoid gelation of the samples. After 10 minutes, the pH was adjusted to 7 and all samples were stored at this pH at -20 °C. The data from repeated hydrolysis experiments (6 times) at several conditions showed that the error in DH (standard deviation/mean x 100) at each point in time is lower than 10 %.

Inactivation Test

The inactivation was verified by hydrolyzing a sample of 5 % (w/v) WPI for 10 minutes using the pH-stat. The pH was afterward brought to pH 2 by addition of 5 M HCl to inactivate the enzyme. After 10 minutes, the pH was readjusted to 8 in the pH-stat. After readjusting the pH to 8, the pH remained constant (for at least 1 hour), indicating that the enzyme had indeed lost its activity.

Determination of the degree of hydrolysis by OPA

The degree of hydrolysis was determined using *o*-phtaldialdehyde (OPA) on samples taken during the hydrolysis with the pH-stat as described in the protein hydrolysis section. The OPA reagent was prepared as described previously [12]. Samples were diluted to 0.5 % (w/v) in a 2 % (w/v) SDS solution, stirred for 20 minutes and stored at

4 °C overnight. The samples were then diluted to 0.2 % (w/v) in Millipore water. Aliquots (5 µL) were added to 300 µL of the reagent solution and equilibrated for 10 minutes. The presence of alkyl-iso-indols formed by the reaction of free amino groups with OPA was measured by the absorbance at 340 nm. To calculate the amount of free NH_2 groups, a calibration curve was measured using leucine as a reference compound.

Solubility

To determine the proportion of soluble proteins in dispersion at 1 to 45 % (w/v), whey protein isolate was weighed and dispersed in Millipore water at 1 to 45 g powder / 100 mL and stirred overnight at 4 °C. The dispersions were then centrifuged (30 min, 4000 g, 20 °C). The protein content in the supernatant was calculated by determining the nitrogen content by Dumas method using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The solubility was calculated as the quantity of protein in the supernatant divided by the initial protein quantity (93.4 % of the initial mass weighed).

Viscosity

The viscosities of protein solutions at concentrations ranging from 1 to 30 % (w/v) were measured using Ubbelohde viscometers with constants (C) varying between 0.005 and 0.1 using a water bath at 25 °C. Viscosities of 1 % (w/v) WPI solutions after addition of glycerol up to 70 % and locust bean gum (LBG) at a concentration of 0.05 % were also determined. Measurements were done in triplicate (standard error < 1 %). The dynamic viscosity (η_{dyn}) in Pa.s was determined by (6).

(6) $$\eta_{dyn} = t \cdot C \cdot \rho \cdot 10^{-6}$$

with t the time to flow in seconds, C the gauge-constant of the Ubbelohde and ρ the density of the solution in $kg.m^{-3}$.

Conductivity

The conductivity of the protein solutions at different concentrations was measured with a conductivity cell (inoLab Cond720, WTW, Weilheim, Germany) at 25 °C [13]. This conductivity was recalculated to ionic strength (equivalent concentration of NaCl) using a calibration curve (equation 7) made by measuring the conductivity of NaCl solutions of concentrations ranging from 0.01 to 0.5 M.

(7) Ionic strength (NaCl equivalent M) = $1.02\ 10^{-5}$ x conductivity ($\mu S.cm^{-1}$).

Size Exclusion Chromatography

To determine if oligomerization took place as a function of initial protein concentration, size exclusion chromatography was performed on an ÄKTA micro system (GE Healthcare, Uppsala, Sweden). A Superdex 75 HR 10/30 column (GE Healthcare) was equilibrated and run at room temperature with a 10 mM potassium phosphate buffer pH

8 and at a flow rate of 800 µL·min^{-1}. Intact proteins were dissolved in 10 mM potassium phosphate buffer pH 8 to concentrations varying from 1 to 30 % (w/v). Samples were centrifuged (10 min, 19000 g, 20 °C), no pellet was observed and 10 µL of the sample were injected onto the column. To test the effect of the presence of NaCl these experiments were also performed in the presence of 0.1 and 0.5 M NaCl (both in the sample preparation and in the running buffer). The detection was performed at 280 nm. Calibration of the column was performed with blue dextran (2000 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa).

For the determination of the proportion of intact protein in the hydrolysates, size exclusion chromatography was performed with the same system using a Shodex protein KW-802.5 column (Showa Denko K. K., Kanagawa, Japan). The eluent was 6 M urea in 30 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA). The flow rate was 600 µL·min^{-1}. The samples were first diluted to 0.5 % (w/v) by addition of a solution containing 6 M urea, 100 mM DTT in 50 mM Tris, HCl, pH 8 and left for incubation for 2 hours at room temperature. ACN and TFA were added to the samples to reach final concentrations of 30 % (w/v) ACN and 0.1 % (w/v) TFA, the final concentration of the samples was 0.1 % (w/v). After centrifugation (10 min, 19000 g, 20 °C), samples (20 µL) were injected onto the column. Detection was performed at 220 nm and 280 nm. The proportion of remaining intact protein for each sample was determined by dividing the area of the protein peak in the sample by the area of the protein peak at DH = 0 (i.e. non-hydrolyzed samples) (using Unicorn software).

RP-UHPLC

Peptide profiles of hydrolysates (DH = 9 %) were analyzed on an Acella UHPLC system (Thermo Scientific, San Jose, CA, USA). ACN and 1 % (v/v) TFA were added to the hydrolysates to reach final concentrations of 5 % ACN (v/v) and 0.03 % TFA (v/v), the final protein concentration was 0.1 % (w/v). Samples were centrifuged (10 min, 19000 g, 20 °C) before injection. Samples (10 µL) were injected on an Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity UPLC BEH C18 Vanguard pre-column (2.1 x 50 mm, 1.7 µm particle size; Waters). Eluent A was 5 % (v/v) acetonitrile (ACN) containing 0.03 % (v/v) TFA and eluent B was 80 % (v/v) ACN containing 0.03 % (v/v) TFA. The elution profile used was: 0-1 min isocratic equilibration with 100 % A, 1-24 min, linear gradient 0-60 % B, a linear gradient 24-26 min, 60-100 % B, 26-29 min isocratic on 100 % B, and 30-34 min isocratic on 100 % A. The flow rate was 300 µL·min^{-1} and the detection was performed at 214 nm. To compare the peptide profiles for each concentration, the chromatograms were divided into three regions according to the retention time: 4-8 min, 8-12 min and 12-18 min. For each region the peak area was calculated and divided by the total area of 4-18 min to determine the proportion of peptides in each region. As was shown previously, the retention time of peptides depends on the molecular weight of the peptides, with shorter

elution times for smaller peptides [14]. Consequently, the three selected regions represent different size classes of peptides.

Results and Discussion

Effects of concentration on hydrolysis kinetics

Suspensions of 1-30 % (w/v) whey protein isolate (WPI) were hydrolyzed by Alcalase at constant enzyme: substrate ratio. During the hydrolysis, the degree of hydrolysis (DH) was followed by the pH-stat method. (**figure 2**)

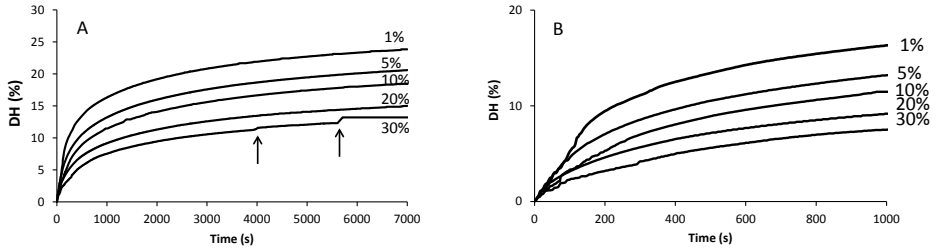

Figure 2. (A) Hydrolysis curves of WPI suspensions hydrolyzed by Alcalase 2.4L at 40 °C for protein concentrations ranging from 1 to 30 % (w/v); arrows indicate effect of gelation. (B) Zoom on 0-1000 seconds.

At 1 % (w/v) WPI a fast initial increase of the DH and a high final DH (24 %) are observed. With increasing substrate concentration, a decrease is observed for both the initial rate of hydrolysis and the final DH. In addition a few irregularities are observed in the curve for the hydrolysis of the 30 % sample, these are due to gelation as a result of the hydrolysis. Similar observations of the decreasing overall rate of hydrolysis with increasing substrate concentration have been described in literature [1,2,4].

Physical properties and hydrolysis kinetics

Solubility and hydrolysis of the proteins solutions

To study parameters that could be involved in the observed decrease in hydrolysis kinetics, several physical properties of the system were determined. (**table 2**) The pH was 7.10-7.20 for all concentrations. The solubility was determined from the protein content in the supernatant after centrifugation of the samples. The solubility decreases from 98 to 78 % for 1 to 30 % (w/v) WPI dispersions. To avoid artifacts due to the presence of nondissolved material the hydrolysis reactions were performed with protein solutions at final concentrations of 1-30 % (w/v) prepared by diluting the supernatant of a 45 % (w/v) dispersion as described in the Materials and Methods section. These protein solutions were analyzed by size-exclusion chromatography (run in 10 mM phosphate buffer with no added NaCl). Even at the higher protein concentrations no change in the elution pattern was observed, showing that there was no formation of higher oligomers or aggregates of the proteins under these conditions (data not shown).

Table 2. Properties of whey protein isolate dispersions.

Concentration % (w/v)	pH ± 0.05	% soluble
1	7.10	98.5
5	7.10	96.4
10	7.20	94.2
20	7.20	85.7
30	7.20	78.5

In the absence of nondissolved material the overall hydrolysis rate and the final DH decreased with an increase in substrate concentration the same way as in the presence of nondissolved material. To compare the two sets of experiments, the overall hydrolysis rate (k_{hydr}) is calculated from the pH-stat results using equation (2) and plotted as a function of the concentration. (**figure 3**) In this figure the results obtained from a different set of hydrolysis experiments with Neutrase (in the absence and in the presence of insoluble material) are also shown.

The DH values reached for Neutrase are lower than those reached with Alcalase. Also, different overall hydrolysis rates are observed for all substrate concentrations. These differences are due to the different activities of the two enzymes. For both enzymes, the overall hydrolysis rate is not affected by the presence of nondissolved proteins. Furthermore, for both enzymes it is observed that the overall hydrolysis rate decreases with increased substrate concentration. (**figure 3**) The removal of the nondissolved proteins (up to 21 % of the total protein concentration) has no significant effect on the kinetics of the hydrolysis. The absence of effects indicates that the nondissolved protein can actually be hydrolyzed by the enzyme.

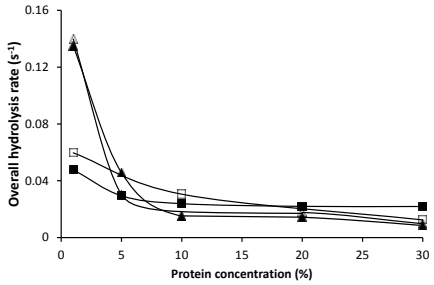

Figure 3. Overall hydrolysis rate as a function of the concentration for dispersions and solutions: WPI hydrolyzed by Alcalase without removal of insoluble parts (▲) and after removal of insoluble parts (△), WPI hydrolyzed by Neutrase without removal of insoluble parts (■) and after removal of insoluble parts (□).

Since the high protein concentration might affect the dissociation constant (α), used to calculate the DH from the pH-stat data, the DH values were also determined from the free amino group concentration using the OPA method. The same DH values were determined using the two methods showing that calculated DH values represent the actual degree of hydrolysis (data not shown).

Since an increase in solution viscosity may influence the hydrolysis, the dynamic viscosities of the protein solutions were determined. The viscosity increases from the viscosity of water (1 mPa·s) at 1 % to only 27 mPa·s for 30 % WPI. (**table 3**) These values are comparable with previously reported values [7]. The viscosity was also determined at 40 °C, which is the hydrolysis temperature and the viscosity is 21 mPa.s for 30 % WPI and 10 mPa·s for 30 % WPI in presence of 0.5 M NaCl.

Table 3. Properties of whey protein solutions, prepared by dilution from the supernatant of a centrifuged 45 % dispersion at 0 and 0.5 M NaCl.

		% Solubility		**η_{dyn} (mPa.s)**		**Conductivity (mS.cm^{-1})**	
Concentration % (w/v)	pH ± 0.05	0 M	0.5 M	0 M	0.5 M	0 M	0.5 M
1	7.10	90	93	0.95	0.74	0.25	44
5	7.10	91	97	1.3	1.17	0.95	43
10	7.20	94	98	2.0	1.62	1.5	41
20	7.20	93	95	5.5	3.00	2.5	34
30	7.20	96	93	27	15.6	3.0	31

To verify if such a small increase in viscosity can explain the large effects on hydrolysis kinetics, the overall hydrolysis rate was determined after increasing the viscosity of a 1 % (w/v) WPI solution by addition of either glycerol (up to 70 %) and locust bean gum (LBG, at a concentration of 0.05 %) to reach viscosities comparable to the viscosity of a 30 % (w/v) WPI solution (25-30 mPa·s). By addition of glycerol the DH reached after 7000 seconds (30 %) is even higher than in the absence of glycerol (24 %). This shows that the viscosity itself does not have a negative influence on the overall hydrolysis rate, nor on the final DH. This was confirmed by the addition of LBG, which reaches a DH of 19 %, which is still high compared to the final DH of the 30 % (w/v) protein hydrolysis (data not shown). These results showed that the increase in viscosity due to higher protein concentrations does not explain the observed effect of slower overall hydrolysis rate with increasing concentration.

Effect of ionic strength on hydrolysis

During characterization of the protein solutions, it was observed that the conductivity of the solutions increases from 0.25 to 3.0 mS·cm^{-1} corresponding to equivalent concentrations of 3 to 30 mM NaCl. (**table 3**) For concentrations < 10 % (w/v) the conductivity increases linearly with the protein concentration, as was previously found for other proteins [13]. At higher concentrations, the conductivity increase levels off. A similar decrease of conductivity at increasing concentrations has been reported for monoelectrolytes, such as NaCl, for concentrations up to 0.2 M [15]. This effect is attributed to a shift in the degree of ionization (dissociation). To avoid differences in the electrostatic interactions resulting from the differences in the ionic strength (i.e. conductivity), proteins were dissolved in 0.1 M NaCl and 0.5 M NaCl.

The conductivity of protein solutions (≤ 10 % (w/v)) in 0.1 M NaCl is close to that of the NaCl solution itself (11 $mS \cdot cm^{-1}$). At higher protein concentrations the conductivity of the protein solution is lower (9.2 and 8 $mS \cdot cm^{-1}$ for 20 and 30 % (w/v) protein) than that of the NaCl solution. Even after a further increase of the NaCl concentration to 0.5 M a similar decrease in conductivity at higher protein concentrations is observed. (**table 3**) Still, at lower protein concentrations, the presence of salt results in a constant conductivity, thus avoiding effects caused by differences in the conductivity. The hydrolysis was then performed at these two ionic strengths. It is important to note that the increase in ionic strength did not result in formation of oligomers, or aggregates of the proteins, as was confirmed by SEC. (**figure 4**) Only a slight shift in the elution time of β-lactoglobulin was observed, indicating the transition in the association state from dimers to trimers. Still, it clearly shows the absence of larger aggregates (> trimers).

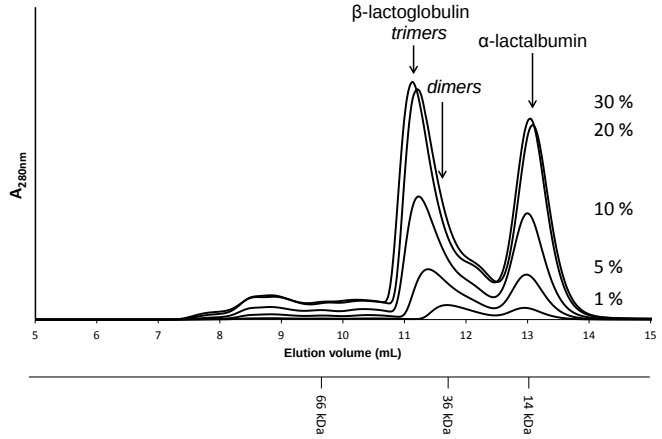

Figure 4. SEC chromatograms of intact WPI injected at different concentrations (1-30 % (w/v), sample and elution buffer contain 10 mM potassium phosphate pH 8.0 and 0.5 M NaCl).

For 1-30 % (w/v) protein solutions (**figure 5A**), the overall hydrolysis rates by Alcalase in the absence and presence of 0.1 M and 0.5 M NaCl were determined. The curves for protein concentrations > 10 % (w/v) are slightly affected by the increased ionic strength; the overall hydrolysis rate (as determined from equation 2) is slightly increasing with increasing ionic strength at these high protein concentrations. At low protein concentrations the increase in ionic strength from 0 to 0.5 M NaCl leads to a significant decrease in kinetics and final DH. For the 1 % protein solution the final DH goes from 24 % at 0 M NaCl to 14 % at 0.5 M NaCl. It is important to note that by the increase of ionic strength the viscosity of this solution was decreased from 0.95 to 0.74 mPa·s. (**table 3**)

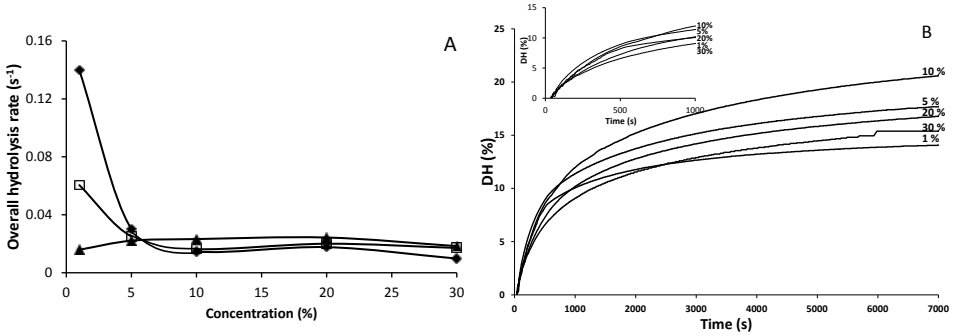

Figure 5. (A) Overall hydrolysis rates of WPI solutions hydrolyzed by Alcalase (at 40 °C) in the presence of 0 M NaCl (♦), 0.1 M NaCl (□), 0.5 M NaCl (▲). (B) Hydrolysis curves of WPI solutions hydrolyzed by Alcalase 2.4L at 40 °C for protein concentrations ranging from 1 to 30 % (w/v) in presence of 0.5 M NaCl. Inset to show initial hydrolysis.

This supports the previous conclusion that the most dominant effects observed are not due to differences in viscosity. Similar significant effects of ionic strength at low protein concentrations have been described for hydrolysis of 0.2 % β-lactoglobulin by trypsin [16]. There, a five times decrease of the overall hydrolysis rate was found when the ionic strength was increased from 0.1 M NaCl to 1.0 M NaCl.

As shown in the inset of **figure 5B**, the initial hydrolysis rates in the presence of 0.5 M NaCl are similar for all protein concentrations, as expected based on equation 1. However, as the hydrolysis proceeds, the curves for different concentrations start to deviate, resulting in differences in the DH at 7000 s. Now, in the presence of 0.5 M NaCl, the final DH increases with increasing concentration from 1-10 %. For 20 and 30 % the final DH decreases with the concentration. As was discussed above, at these concentrations, the conductivity is also not equal to that of the NaCl solution. This indicates that the presence of 0.5 M NaCl is still not sufficient to avoid effects due to differences in conductivity. Further increase of NaCl concentration to 0.7 M showed a further increase in the final DH of 20 and 25 % protein. Still, at this condition (46 $mS \cdot cm^{-1}$) the final DH did not exceed that of the 10 % at 0.5 M NaCl (46 $mS \cdot cm^{-1}$).

Summarizing, starting from a 1 % WPI solution in water an increase in conductivity, either by increasing NaCl (0.5 M NaCl), or by increasing protein concentration (to 30 %), results in a decrease in the overall hydrolysis rate, and in the final DH. While the decrease in overall hydrolysis rate is similar in both cases, the conductivity is not (44 $mS.cm^{-1}$ for 1 % in 0.5 M NaCl and 3.0 $mS \cdot cm^{-1}$ for 30 % in water). Furthermore, in the presence of 0.5 M NaCl, an increase in protein concentration (from 1 to 10 %) does not significantly affect the overall hydrolysis rate, but it does result in an increase in the final DH.

Hydrolysate composition

In the previous section, the hydrolysis kinetics, based on the degree of hydrolysis were discussed. To characterize the hydrolysis in more detail, in this section the composition

of the hydrolysates is described with respect to the remaining amount of intact protein and the peptide profile.

Degradation of intact protein

The amount of intact β-lactoglobulin (compared to the initial amount) determined by SEC is plotted versus the degree of hydrolysis. (**figure 6**) This plot can be used to distinguish if the observed differences in kinetics are only due to kinetics, but also due to differences in the mechanism of hydrolysis. The results show two sets of samples with similar behavior. (**figure 6A**) At low concentrations (1-10 % (w/v)) the amount of intact protein decreases to 30 % of the initial amount at DH 3 %. For 20 and 30 % (w/v) the DH to reach 30 % intact protein is DH 4.5 %. This indicates that at higher substrate concentrations the enzyme has a slightly higher activity toward the fragments than toward the intact protein. In presence of 0.5 M NaCl, (**figure 6B**) the same two sets of samples can be differentiated. For 5 and 10 % protein, a deviation in the curve is observed between DH 2 % and DH 5 %. This might be due to large peptides coeluting with β-lactoglobulin which affect the measured area under the curve and thus the proportion of intact remaining β-lactoglobulin.

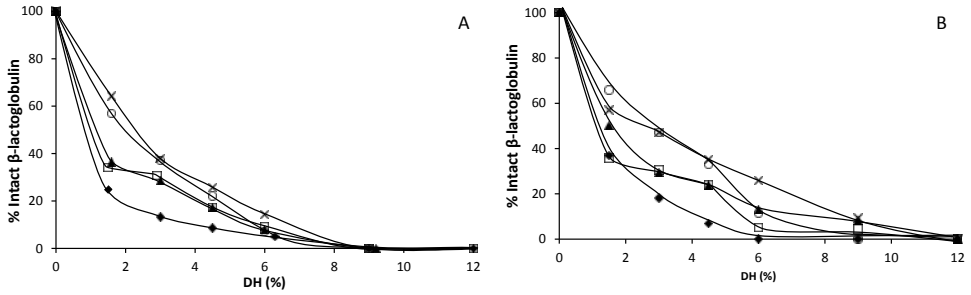

Figure 6. Proportion of intact β-lactoglobulin as a function of the degree of hydrolysis at different initial protein concentrations. (A) for the proteins solutions in 0 M NaCl, (B) for the protein solutions in 0.5 M NaCl. At different initial protein concentrations: (♦) 1 %, (□) 5 %, (▲) 10 %, (**X**) 20 %, (○) 30 %.

Still, the results indicate a higher activity of the enzyme toward intact β-lactoglobulin for low concentrations and toward fragments for higher concentration. A clear difference between the hydrolysis in the presence and absence of salt is observed in the DH at which all of the intact protein is hydrolyzed. In the absence of salt, the protein is entirely hydrolyzed at DH 9 % for all initial protein concentration while in the presence of 0.5 M NaCl, β-lactoglobulin is completely hydrolyzed at DH 6 % for 1 % and at DH 12 % for the other concentrations.

Peptide profile

RP-UHPLC chromatograms of the hydrolysates were measured for the different protein concentrations at the same DH (9 %). If the enzyme would have the same action, the

peptide profile at a given DH should be similar. To compare the peptide profiles, the chromatograms were divided in sections (**figure 7**) and the relative peak area in each section was determined.

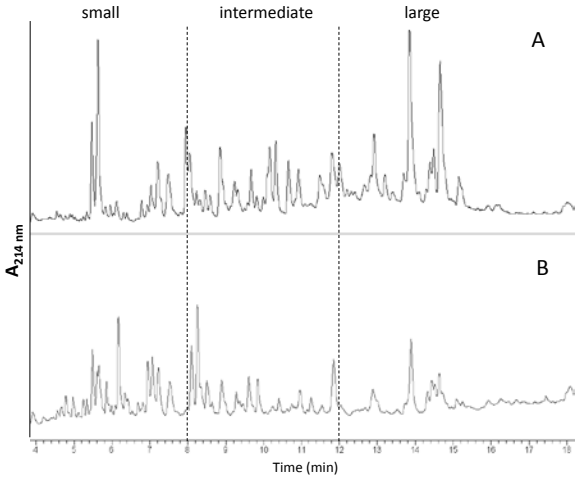

Figure 7. RP-UHPLC chromatograms of the hydrolysates of 1 % initial protein concentration at DH = 9 % at (A) 0 M NaCl and (B) 0.5 M NaCl.

From this the proportions of small, intermediate and large peptides were calculated. (**figure 8**) At 0 M NaCl, a slow continuous change in the peptide profile is seen with increasing substrate concentration. The relative amount of small peptides (at short retention times) increases, while the amount of larger peptides (at high retention times) slightly decreases. In another study, a decrease in the amount of hydrophobic peptides with increasing substrate concentration was also observed for whey protein isolate hydrolysates prepared at 5-30 % (w/v) [17].

At 0.5 M NaCl compared to 0 M NaCl, the composition of the 1-5 % samples shows an increase in hydrophilic peptides, and a decrease in the amount of hydrophobic peptides. At 0.5 M NaCl, the final composition at 1 % protein becomes more similar to the sample at 30 %. The results obtained from the peptide profile analysis show a certain similarity with the kinetics of the hydrolysis. At higher ionic strength the hydrolysate of the 1 % (w/v) solution changes to the direction of the hydrolysate of the 30 % WPI solution, while the intermediate samples (10-20 %) remain more or less constant. In the absence of NaCl, the proportion of large peptides decreases with increase of substrate concentration. Summarizing, at 0 M NaCl smaller peptides are formed with increasing substrate concentration At 0.5 M NaCl, the proportion of large and/or hydrophobic peptides decreases at all substrate concentrations, in comparison with hydrolysis at 0 M NaCl [14].

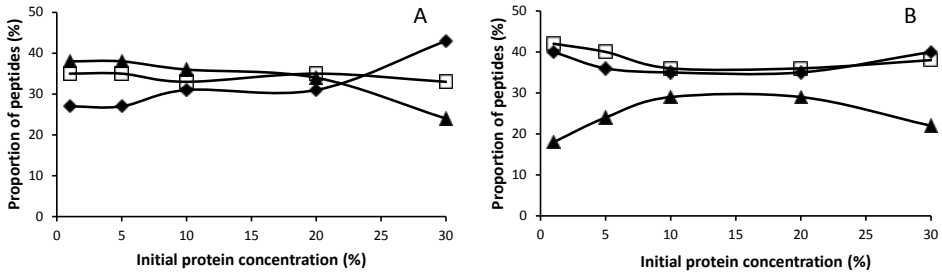

Figure 8. Proportion of (♦) small, (□) intermediate and (▲) large peptides in the hydrolysates as a function of the initial protein concentration at (A) DH = 9 % at 0 M NaCl and (B) 0.5 M NaCl.

Effects of ionic strength on WPI and on the hydrolysis

The decrease of enzymatic protein hydrolysis kinetics with increasing concentration of NaCl has been observed in earlier experiments on hydrolysis of β-lactoglobulin with trypsin [16], but was not linked in later studies to the observed decrease with increasing substrate concentration. It was proposed that this decreased overall hydrolysis rate with increasing ionic strength could be due to increased structural stability of the protein [16]. The increase of structural stability at higher ionic strength has indeed been shown for β-lactoglobulin [18] and no significant effect was observed for whey protein isolate (WPI) [19]. An increase from 0 to 0.5 M NaCl resulted in an increase of the denaturation temperature of β-lactoglobulin (14 % (w/v)) by 6 °C [18]. The thermal stabilities with increasing WPI concentration from 2 % to 10 % were found to be similar [19], while the overall hydrolysis rates are clearly not. (**figure 5A**) This shows, that the differences in overall hydrolysis rate due to differences in concentration of protein or NaCl cannot be directly attributed to differences in thermal stability. This is also illustrated by the fact that for small peptides, that do not have defined secondary or tertiary structures, different effects of ionic strength on hydrolysis rates have been observed. An increase in ionic strength (0-0.9 M NaCl) had no significant effect on the rate of hydrolysis of synthetic peptides by Alcalase [20], but resulted in an increased rate of hydrolysis of synthetic peptide substrates by thermolysin [21].

It has been shown that the hydrolysis of synthetic peptides by Alcalase is not affected by NaCl concentrations from 0-0.9 M NaCl [20]. Therefore, it is concluded that the decreased overall hydrolysis rate by increased conductivity (by increasing NaCl or protein concentrations) is not the result of decreased enzyme activity. It was further shown, that the effect cannot be attributed to changes in viscosity, thermal stability [19] or aggregation state of the protein. So, while it is very clear that the conductivity and NaCl concentration influence the hydrolysis kinetics, the exact mechanism is not yet understood.

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Chapter 3

Influence of water availability on the enzymatic hydrolysis of proteins

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Abstract

The overall rate of enzymatic hydrolysis of proteins is found to decrease with increasing protein concentration (0.1-30 % (w/v)) at constant enzyme/substrate ratio. To increase the insight into the underlying mechanism, the role of water on the hydrolysis was studied. The available water was calculated as the ratio between free and bound water, and additionally determined by water activity measurements and from T_2 relaxation time measurements using NMR. At low protein concentrations a large excess of water is present ($1.53 \cdot 10^6$ water molecules per protein molecule at 0.1 % (w/v) whey protein isolate (WPI), but only 3,984 water molecules per protein at 30 % (w/v) WPI. Assuming that around 357 molecules of water are needed for full hydration of the protein, these values correspond to a 4,280 and 11 times excess of water, showing that at 30 % (w/v) WPI the amount of water becomes limited. Since the release of charged residues after hydrolysis of the peptide bond would require additional hydration water, the limitation of water is considered to increase during hydrolysis. Still, measurement of the water activity showed only a decrease from 1.00 to 0.96 (for 0.1 % and 30 % (w/v) WPI). From the determination of T_2 relaxation times with NMR, an increase in the proportion of bound water is determined, to less than 1 % of bound water at 0.1 % (w/v) WPI up to 10 % of bound water at 30 % (w/v) WPI. As an indication of the availability of water in the solution, the free to bound water ratio was calculated at different conditions. It was shown that the decrease in free to bound water ratio of the solution, either by increasing the protein concentration or by addition of co-solutes (e.g. glucose, or 0.5 M NaCl) correlates with a lower initial hydrolysis rate and a lower DH reached.

Submitted

Introduction

Water availability in protein solutions

In studies on protein hydrolysis typically quite dilute substrate solutions or suspensions (i.e. < 10 % (w/v) protein) are used. While increasing the protein concentration (e.g. up to 30 %) would allow a decrease in the consumption of water in industrial processes it may also hinder the hydrolysis. It is generally observed that increasing substrate concentrations result in lower hydrolysis rate [1]. The reason for the effects of increased concentrations on the enzymatic hydrolysis are quite unclear. Increasing the protein concentration from 0.1 to 30 % (w/v) with a specific volume of the protein of 0.73 $cm^3 \cdot g^{-1}$ [2], results in a decrease in the concentration of water from 55 M to 42 M. This means that the molar ratio of water per protein decreases. For example, for a 0.1 % (w/v) β-lactoglobulin solution there are 945,500 molecules of water per protein molecule, while for a 30 % (w/v) β-lactoglobulin solution only 2,650. To assess if this is an excess amount of water, a comparison is made with the amount of hydration water needed for the different amino acids. Based both on experiments and calculations the hydration layer of a monomeric protein in its folded state has been reported to correspond to approximately 0.35 g water per g of protein [2]. For β-lactoglobulin, this would mean that the hydration number is 357 moles of water per mole of protein. This means that at 10 and 30 % (w/v) protein, the available amount of water is 26 and 7 times the amount needed for hydration of the native protein, respectively. This already illustrates that there is no large excess of water. For a fully denatured protein, considering the different hydration of the amino acid residues [3] and the amino acid composition of β-lactoglobulin (www.uniprot.org - accession number P02754), 439 moles of water are necessary to hydrate one mole of β-lactoglobulin, equivalent to 0.43 g H_2O per g protein. These values show that the amount of hydration water does not depend much on the folding state of the protein. This is because most water is associated with the amino acids with charged side chains that are present mostly on the outside of the native protein. On average, 5 water molecules are necessary per charged side chain residue, while 2 are needed for the other amino acids [3]. During enzymatic protein hydrolysis, the number of charged groups is increased, since each cleavage leads to the formation of one additional NH_3^+ and one COO^- group at pH values between 4 and 9. Consequently, an increasing amount of water is required for hydration during hydrolysis. The hydration water associated with the amino acids is qualified as bound water opposite to the free water present in the bulk. An indication of the proportion of free water is typically obtained by measurements of the water activity (a_w). It is assumed that water activity depends mostly on ions present [4]. Water activity depends on the molar fraction of water and on the activity coefficient of water, which itself depends on the ionic strength. The water activity of NaCl solutions for instance, decreases linearly with increasing concentration [5]. The fractions of free and bound water in protein solutions can be quantified from the relaxation time of water as determined by NMR [6]. Free water, measured from the relaxation time is correlated linearly with increasing protein concentration. Above 280 $g \cdot L^{-1}$, the relaxation rate and

protein concentration follow a different linear relationship than below 280 $g \cdot L^{-1}$ [7], indicating a change in the water behavior at high protein concentrations. Above 280 $g \cdot L^{-1}$, water molecules are probably slowed down as a result of increasing viscosity. In another example, the T_2 relaxation rate of the bulk water of cellulose suspensions decreases from 600 ms to 200 ms by increasing the concentration from 5 % to 15 % (w/w). A similar decrease is observed when 1 % (w/v) glucose is added to a 5 % (w/w) cellulose suspension. This indicates that increasing the concentration of cellulose or adding glucose have an influence on the water behavior [8].

Influence of substrate concentration and water on the hydrolysis

It was previously shown that enzymatic hydrolysis performed at increasing substrate concentration or limited water content leads to lower rates of hydrolysis and lower apparent final DH values (at long times) even in the range 1-10 % (w/v). This was observed for different substrates, such as whey proteins [1] and wheat gluten [9] at constant enzyme to substrate ratio and also for non-protein systems such as cellulose [10]. It has previously been suggested that hydrolysis at higher substrate concentration is slowed down due to diffusion limitations. The rate of hydrolysis of WPI was, however, not affected when the viscosity of a 1 % (w/v) solution was adjusted to that of 30 % (w/v) solution (27 mPa·s) by addition of 0.05 % (w/v) locust bean gum [1]. This shows that the viscosity does not play a significant role in the decrease of hydrolysis with increasing protein concentration. An alternative explanation for the decreased rate of hydrolysis at increased protein concentration is substrate inhibition [11,12]. Substrate inhibition is assumed to be the result of binding of the substrate to the enzyme outside of the active site. Substrate inhibition has been described for various substrate/enzyme systems at very low substrate concentrations. It occurs, for example, at concentrations of 100-250 µM (0.002-0.005 % (w/v)) of tryptophan and tyrosine hydrolyzed by tryptophan and tyrosine hydroxylase [13], or 0.28 µM (0.04 % (w/v)) hyaluronan hydrolyzed by hyaluronidase [14]. For proteases, substrate inhibition has been described for concentrations above 10 µM for peptides hydrolyzed by a cysteine protease [15] and above 100 µM for azocasein hydrolyzed by savinase [16]. In this last example the enzyme activity decreases at substrate concentrations between 100 µM to 350 µM, (0.24-0.83 % (w/v)), and remains constant (up to 600 µM or 1.4 % (w/v)). The reported substrate inhibition curves typically tend to reach a plateau at low concentrations (< 350 µM or ≤ 0.04 % (w/v)). It is important to note that the concentrations where substrate inhibition is reported are quite low compared to the protein concentrations where decrease of the hydrolysis rate was observed (from 1 to 30 % (w/v), equivalent to 360 µM to $10 \cdot 10^3$ µM for WPI). In these latter systems, all concentrations are well above the different reported ranges for substrate inhibition, while still a further decrease in rate of hydrolysis is observed. Hence, a different mechanism is probably responsible for the decrease in hydrolysis rate observed when increasing the substrate concentration from 1 to 30 % (w/v) protein. A possible reason for the further decrease in the hydrolysis rate (and final DH) is the lower water

concentration and the non-ideality of the system. It has, for instance, been shown that the pK_a values of dissociating groups start deviating from those in dilute (ideal) systems when the concentrations are increased (non-ideal systems). This was observed in studies at increasing concentrations of NaOH, where the observed electrode potential deviates from ideal behavior (i.e. linearity) for concentrations above 0.1 M [17]. Similarly, it was observed that the conductivity of WPI solutions deviates from the linearity above 10 % (w/v) WPI (equivalent to 3.6 mM WPI or 0.2 M charged groups). The deviation from linearity is observed at a lower protein concentration than for deviation from linearity of the relaxation rate determined by NMR. This is probably the result of two different effects of the increase in protein concentration on the proteins.
While an increase in substrate concentration automatically results in a lower amount of available water, the exact effects of water availability on protein hydrolysis have not been studied in detail. To increase the understanding of the influence of water on protein hydrolysis, in this study the changes of the properties of the system are characterized with respect to water activity and availability. These effects were tested by increasing protein concentrations as well as additions of co-solutes, such as glucose and NaCl.

Material and Methods

Materials Bipro, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). The protein composition (by weight) was 74.0 % β-lactoglobulin, 12.5 % α-lactalbumin, 5.5 % bovine serum albumin and 5.5 % immunoglobulin according to specifications of the supplier. The protein content of the powder was 93.4 % (w/w), determined by Dumas (Nx6.32 calculated as described before [1]). Alcalase 2.4L (Subtilisin A from *Bacillus licheniformis*, batch PMN 05087), Neutrase 0.8L (*Bacillus amyloliquefaciens,* batch PWN 10034) and BLP (*Bacillus licheniformis* protease) (batch NS-37005) were obtained from Novozymes (Bagsvaerd, Denmark). The BLP had a protein content of 4.5 % (w/w) protein as determined by the Dumas method (Nx6.25) and an activity of 0.3 AU/mg/min as determined by the azocasein assay at 40 °C and pH 8 [18]. Since it was partly insoluble, BLP was fractionated by first solubilizing the BLP powder in 10 mM NaH_2PO_4 pH 5.8 and stirred overnight at 4 °C. The suspension was centrifuged (10 min, 4000 g, 25 °C). The supernatant obtained was extensively dialyzed against 150 mM NaCl, followed by dialysis against demineralized water using cellulose dialysis membranes (cut-off 12-14 kDa). The freeze dried material was found to contain 60 % (w/w) protein (Nx6.25) and an activity of 3.9 AU/mg/min was determined by azocasein assay. It was determined by RP-UPLC and identification by MS, based on UV_{214} that about 78 % of the total UV area corresponds to the enzyme BLP (23.6 kDa, uniprot accession number P80057) and 14 % to the pro-peptide (6.9 kDa). All other chemicals were of analytical grade and purchased from Sigma or Merck.

Hydrolysis

Hydrolysis by different enzymes

Protein solutions were prepared by dispersing WPI powder at a concentration of 45 % (w/v), followed by stirring overnight at 4 °C. Insoluble parts were removed by centrifugation (30 min, 4000 g, 20°C) and the supernatant was diluted to the required final concentrations (0.1-30 % (w/v)) based on UV_{280} absorbance as described before [1]. Solutions of WPI at 0.1, 0.5, 1, 5, 10, 20 and 30 % (w/v) were hydrolyzed using a pH-stat. Separate hydrolyses were performed with three different enzymes (Alcalase, Neutrase and BLP). Alcalase and Neutrase are enzymes with broad specificity, while BLP is specific for glutamic and aspartic acids. The protein solutions (10 mL) were preheated 15 minutes at 40 °C (for Alcalase and BLP) or 50 °C (for Neutrase) and adjusted to pH 7 (Neutrase) or 8 (Alcalase and BLP) before addition of the enzymes. Per mg of protein, 0.13 µL (Alcalase, or Neutrase) or 0.30 µL of a fresh 5 % (w/v) solution of BLP in water was added. For all experiments, NaOH was added to the protein solutions during the hydrolysis to keep the pH constant. Different concentrations of NaOH were used for the different initial protein concentrations, ranging from 0.02 M NaOH for 0.1 % WPI to 6 M NaOH for 30 % (w/v) WPI.

During hydrolysis with Alcalase and BLP, samples were taken at DH 1.5, 3, 4.5, 6, 7 and 9 %. The enzyme was inactivated by adjusting the pH of the solutions to pH 2 with 5 M HCl. After at least 10 min, the pH was re-adjusted to pH 8.0 with NaOH before storage at -20 °C. The inactivated samples were used for the determination of the degree of hydrolysis by OPA. For determination of the water activity, hydrolysates obtained after 8,000 s of hydrolysis with Alcalase were used directly after hydrolysis, i.e. without inactivation.

Hydrolysis in presence of co-solutes

To determine the influence of the solvent quality on the hydrolysis, NaCl, glucose or proline were dissolved in the protein solutions at least 2 hours before enzymatic incubation. NaCl was dissolved to a final concentration of 6 M in the different protein solutions (1 %, 10 % and 30 % (w/v)). Glucose was added to a 1 % or 10 % (w/v) WPI (to final concentrations of 29 and 20 % (w/v), or 1.6 and 1.1 M, respectively), to reach total dry matter concentrations of 30 % (w/v). Proline was dissolved to a final concentration of 1 M in 1 % (w/v) WPI. The effects of the presence of co-solutes on the hydrolysis were tested with Alcalase under the same conditions as described for protein solutions alone. The results obtained in this study were compared with the DH reached and initial rate of hydrolysis of 1 % WPI in the presence of 0.5 M NaCl obtained in a previous study [1].

Characterization of the hydrolysis

The degree of hydrolysis (DH) was calculated from the volume of NaOH (V_b) added during the hydrolysis to keep the pH constant, using equation (1) as described before [1].

(1) $$DH\ (\%) = V_b \times N_b \times \frac{1}{\alpha} \times \frac{1}{M_p} \times \frac{1}{h_{tot}} \times 100$$

In which h_{tot} is the total number of peptide bonds per gram protein substrate. h_{tot}(WPI) = 8.5 as described before [1]. α is the average degree of dissociation of α-amino groups or α-carboxylic groups in a protein. α is calculated using equation (2)

(2) $$\alpha = \frac{1}{1+10^{(pK_{a,NH_2}-pH)}} - \frac{1}{1+10^{(pH-pK_{a,COOH})}}$$

where pH is the pH of hydrolysis, $pK_{a,NH2}$ the average dissociation constant of α-amino groups and $pK_{a,COOH}$ the average dissociation constant of α-carboxylic groups in a protein [19]. The average pKa of the α-amino groups in a protein is calculated with (3)

(3) $$pK_{a,NH_2} = 7.8 + \frac{(298-T)}{298\times T} \times 2400$$

In which T is the temperature in Kelvin [20]. The typical pK_a of α-carboxyl groups is 3.1 at 25 °C [21] but no equivalent to equation 3 was found in literature.

The rate of hydrolysis was described in three different ways. The initial rate of hydrolysis, $k_{initial}$ is determined from the slope of the linear portion of the hydrolysis curves (0-100 seconds). The overall hydrolysis rate k_{hydr} was obtained by fitting equation (4) to the curve of DH versus time.

(4) $$DH = \frac{1}{a}\ln(1 + k_{hydr}.t)$$

in which k_{hydr} (s^{-1}) and a are fitting parameters for overall hydrolysis rate and substrate inactivation, respectively, and t the time (s) [1]. To compare results of hydrolyses by different enzymes, the relative overall hydrolysis rate was calculated using the highest overall hydrolysis rate as 100 % and adapting the other values proportionally.

To analyze the effect of the available amount of water on the hydrolysis, the local rate of hydrolysis (dDH/dt) at each time point is determined by calculating the slope of the DH curve at each time point.

Determination of the degree of hydrolysis by OPA.

The degree of hydrolysis of samples taken during the hydrolysis with the pH-stat was verified using *o*-phtaldialdehyde (OPA). The OPA reagent was prepared as described previously [22]. Samples were prepared as described previously [1] to a final concentration of 0.2 % (w/v). Aliquots (5 µL) were added to 300 µL of the reagent solution and equilibrated for 10 minutes. The presence of alkyl-iso-indols formed by the reaction of free amino groups with OPA was measured by the absorbance at 340 nm. To calculate the amount of free NH_2 groups, a calibration curve was determined using leucine as a reference compound.

Calculation of the free/bound water ratio

To characterize the availability of water in the solutions, five parameters are calculated. As first indications, the available amount of water is expressed as the number of water molecules per protein, or as the number of water molecules per amino acid. To describe how much water is needed for hydration, the fraction of bound water needed

for hydration is calculated. From this the fraction of free water is calculated. The excess amount of water is then obtained by calculating the free water over bound water ratio. First, the initial concentration of water in the solution $C^i_{H_2O}$ (mol·L^{-1}) is calculated by (5)

(5) $$C^i_{H_2O} = \frac{m_{solution} - \upsilon_{protein} \times m_{protein} - \upsilon_{co-solutes} \times m_{co-solutes}}{M_{H_2O}}$$

In which $m_{solution}$, $m_{protein}$ and $m_{co\text{-}solutes}$ are the masses of the solution, of the protein and of the co-solutes (glucose, NaCl and proline), respectively, in the solution in g. $\upsilon_{protein}$ is the specific volume of the protein (0.73 mL·g^{-1} for β-lactoglobulin [2]). $\upsilon_{co\text{-}solutes}$ is the specific volume of the co-solutes: 0.62 mL·g^{-1} for glucose [23]; 0.70 mL·g^{-1} for proline [24] and 0.3 mL·g^{-1} for NaCl [25].

The concentration of water $C_{H2O,DH}$ (mol·L^{-1}) present in the solution as a function of the DH is calculated using equation (6)

(6) $$C_{H_2O,DH} = C^i_{H_2O} - \underbrace{\frac{DH}{100} \cdot \#_{bonds} \cdot C_{protein}}_{\text{Water consumed for hydrolysis}}$$

With $\#_{bonds}$ the number of mole of bonds in one mole of protein, DH the degree of hydrolysis, $C_{protein}$ the concentration of protein in mol·L^{-1} and $C^i_{H_2O}$ the initial concentration of water in the solution in mol·L^{-1}, as determined by equation (5).

The composition of whey protein isolate is described in **table 1**. For practical purposes, a theoretical protein based on the characteristics and proportions present of the constituting proteins was defined. This theoretical protein WPI has a molecular mass of 27,549 g·mol^{-1}, and contains 239 amino acids.

Table 1. Composition of whey protein isolate (Bipro) [1].

	Uniprot no.	% (w/w) in Bipro	M_w (g·mol^{-1})	# of amino acids	# amino acids with charged side chains
β-Lactoglobulin	P02754	74	18,281	162	44
α-Lactalbumin	P00711	12.5	14,186	123	33
Bovine serum albumin	P02769	5.5	66,432	583	181
IgG1	na[a]	5.5	145,000	1218[b]	365[b]
Theoretical WPI			27,549	239	68

[a]Not available. [b]Since no reported sequence was available, the number of amino acids and the number of amino acid with a charged side chains was calculated using the average molecular weight of an anhydro amino acid (119 g·mol^{-1})

Indications of the water availability are determined by calculating the number of water molecules per protein by dividing the initial concentration of water in the solution $C^i_{H_2O}$ (mol·L^{-1}) by the concentration of protein $C_{protein}$ (mol·L^{-1})

(7) $$number\ of\ water\ molecules\ per\ protein = \frac{C^i_{H_2O}}{C_{protein}}$$

The second parameter of water availability is obtained by calculating the number of water molecules per amino acid in the protein:

(8) $$number\ of\ water\ molecules\ per\ amino\ acid = \frac{C^i_{H_2O}}{C_{protein} \times \#AA}$$

In which $C^i_{H_2O}$ (mol·L^{-1}) and $C_{protein}$ (mol·L^{-1}) are the concentration of water and protein in the solution, respectively. #AA is the number of moles of amino acids in one mole of protein.

For the third parameter, the hydration of the theoretical WPI is determined more precisely based on the amino acid composition and the hydration number of individual amino acid residue [3]. It was calculated that 657 moles of water are needed to hydrate one theoretical mole of WPI. In addition, the hydration of the terminal groups and the hydration of the groups formed for each bond cleaved is calculated from the hydration of a COOH group and a NH_2 group. One mole of COOH groups is hydrated by 6.8 moles of water (as described for acidic amino acid residues [3]) and a mole of NH_2 groups is hydrated by 4.5 moles of water (as described for lysine residues [3]). This gives an average hydration of 5.65 moles of water per mole of released or terminal group [3].
From this, the concentration of bound water $C_{bound\ water}$ (mol·L^{-1}) can be calculated as a function of the DH using equation (9):

(9) $$C_{bound\ water} = \underbrace{\frac{DH}{100} \cdot \#_{bonds} \cdot C_{protein} \cdot 5.65 \cdot 2}_{\text{Hydration charges - bonds cleaved}} + \overbrace{657 \cdot C_{protein}}^{\text{Hydration amino acid residues}} + \underbrace{2 \cdot 5.65 \cdot C_{protein}}_{\text{Hydration terminal groups}} + \overbrace{n_{H,cosolutes} \cdot C_{co-solutes}}^{\text{Hydration co-solutes}}$$

In which $\#_{bonds}$ is the number of moles of bonds in one mole of protein, DH the degree of hydrolysis (%), $C_{protein}$ the concentration of protein (mol·L^{-1}). $C_{co\text{-}solutes}$ is the concentration (mol·L^{-1}) of co-solutes and $n_{H,co\text{-}solutes}$ (mol of water per mole of solute) is the hydration number for the co-solutes: 4.5 for NaCl [26], 3.45 for glucose [27] and 2.8 for proline [28]. The minor effects of charges and volume due to the addition of NaOH during the hydrolysis are neglected in these calculations.
The fraction of free water is calculated as

(10) Fraction free water = ($C_{H_2O,DH}$ – $C_{bound\ water}$)/ $C_{H_2O,DH}$

With $C_{H2O,DH}$, the concentration of water (mol·L^{-1}) at each DH from equation 6.

Finally, the free to bound water ratio is calculated as

(11) Free/bound water ratio = fraction free water / (1 - fraction free water)

Water activity measurement

Water activity (a_w) measurements were performed on an Aqualab Meter 4TE (Decagon Devices, Pullman, WA, USA) with an automatic equilibration time of 5 - 10 minutes. All samples were measured in duplicate at 40 °C.
The change in water activity during hydrolysis (expressed as Δa_w) is calculated by:

(12) $$\Delta a_w = a_w(initial) - a_w(8{,}000\ s\ of\ hydrolysis).$$

Free water measurements by NMR

T_2 relaxation measurements were performed for protein concentrations of 0.1-30 % (w/v) WPI. Measurements were performed with 4 acquisitions, τ=500 µs, dwell of 10 µs using a relaxation delay of 10 s and a number of echo of 10 or 8 or 4 K with 5 data points acquisition per echo.

The fraction of free water was obtained from the relaxation rate using equation (13)

(13) $$\frac{1}{T_2 obs} = \frac{F_{bound}}{T_2 bound} - \frac{F_{free}}{T_2 free}$$

In which $T_{2,\ obs}$ is the relaxation time measured for water. F_{bound} and F_{free} indicate the fractions of bound and free water.

Results and Discussion

Initial protein solutions

Hydrolysis of whey proteins

A decrease of the degree of hydrolysis (DH) is observed with increasing protein concentration from 0.1 % to 30 % (w/v) WPI hydrolyzed by Alcalase at constant enzyme:substrate ratio (E/S) after comparable hydrolysis time (8,000 s). (**figure 1**)

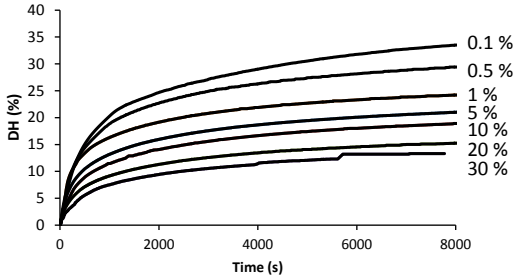

Figure 1. Hydrolysis curves of 0.1-30 % (w/v) WPI hydrolyzed by Alcalase at constant enzyme/substrate ratio.

The relative overall hydrolysis rate shows an exponential decrease with increasing substrate concentration. (**figure 2A**) The same trend was observed when the hydrolysis was performed with the other a-specific enzyme Neutrase and the specific enzyme BLP. (**figure 2**) This indicates that the decrease in hydrolysis rate with increasing substrate concentration does not depend on the enzyme or enzyme specificity. A difference is, however, observed in the gelation that occurs during the hydrolysis at high protein concentrations. For Neutrase and Alcalase the 30 % (w/v) WPI hydrolysate gels after 8,000 s, while for BLP gelation occurs in the first minutes at concentrations of 20 and 30 % (w/v) WPI. Therefore, data for BLP were only obtained for concentrations up to 10 % (w/v).

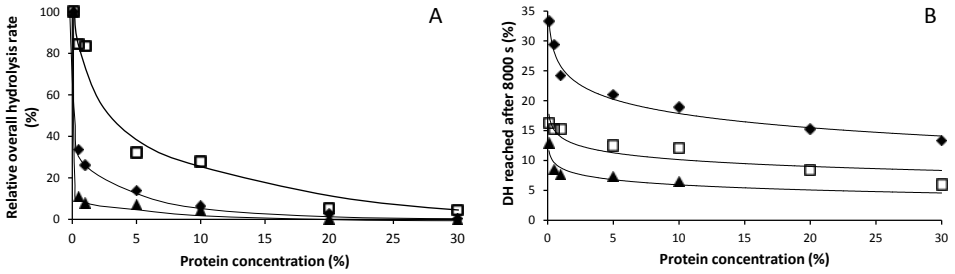

Figure 2. (A) Relative overall hydrolysis rate and (B) DH reached after 8,000 s of hydrolysis as a function of the initial substrate concentration for hydrolysis of WPI by three different enzymes, (♦) Alcalase, (□) Neutrase and (▲) BLP.

For all enzymes, the final DH (reached after 8,000 s) decreases with increasing protein concentration, similarly as observed for the overall hydrolysis rate. (**figure 2B**) Of course, the absolute values of the final DH are different for the three enzymes because of differences in specificity. Similar observations have been obtained for, e.g. rapeseed protein at constant enzyme concentration [29] and for whey proteins at constant enzyme/substrate ratio [11].

This shows that the effect of concentration is independent of the enzyme and the substrate. Still, at increasing protein concentration and constant enzyme concentration, a proportional decrease in the overall hydrolysis rate is expected, with increasing protein concentration. At constant enzyme/substrate ratio, an increase in the number of cleaved bonds is expected which translates in an overall hydrolysis rate independent of the concentration [1]. Surprisingly, only for tryptic hydrolysis of BSA (0.3-0.8 $g \cdot L^{-1}$) an increase in overall hydrolysis rate has been observed with increasing protein concentration at constant enzyme concentration [30].

To confirm the DH values obtained with the pH-stat method, the DH was measured using the OPA method. (**figure 3**) With both methods, the same DH values were determined for hydrolysates obtained with Alcalase up to DH 9 % and for BLP up to DH 6 %. (**figure 3**) The last DH point obtained during hydrolysis with BLP for protein concentrations of 5 and 10 % WPI gives a lower value by OPA than by the pH-stat. A lower DH by OPA than by pH-stat was also observed at high DH for the hydrolysis with Alcalase. (data not shown) This shows an overestimation of the DH by the pH-stat at high DH. The overestimation of the DH is not likely to be caused by the pK_a values of α-amino groups, as these are described to increase with a decrease of the chain length [31], since this would lead to an underestimation. Overall, the DH calculated by the pH-stat and by OPA are comparable and the increasing protein concentration does not affect the value of the DH measured by the pH-stat.

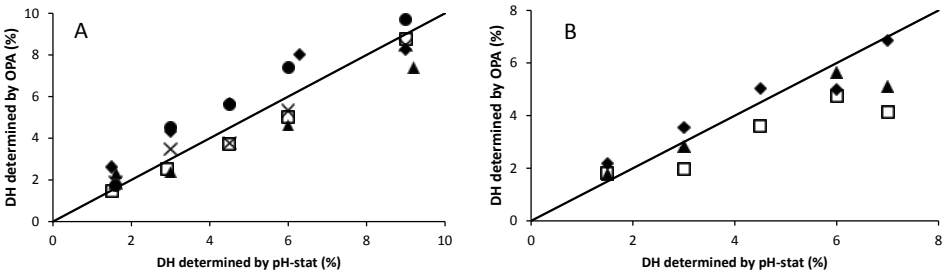

Figure 3. Comparison of the DH value obtained by the pH-stat method and by OPA method for different initial WPI concentrations (♦) 1 %; (□) 5 %; (▲) 10 %; (X) 20 %; (●) 30 % for two enzymes (A) Alcalase and (B) BLP.

Water availability

In a 0.1 % (w/v) WPI solution there are $1.5 \cdot 10^6$ water molecules per protein molecule and at 30 % (w/v) WPI 3,984. (equation 7) In other words, for a 0.1 % (w/v) protein solution, there are 6,380 water molecules per amino acid (equation 8) and at 30 % (w/v) WPI solution only 16. (**table 2**) This gives an indication of the excess of water at low protein concentration and of the limited amount of water in the solution at high protein concentration. To determine the availability of the water in the solution, the fraction of free water is calculated (equation 10). The fraction of free water is 0.99 for 0.1 % (w/v) WPI solution and 0.83 for 30 % (w/v) WPI. (**table 2**) This shows a decrease of 20 % of the fraction of free water by increasing the protein concentration 300 times.

Table 2. Initial amount of free and bound water at increasing protein concentrations (0.1-30 % (w/v) WPI).

Protein concentration % (w/v)	Number of water molecules per protein	Number of water molecules per amino acid	Fraction of free water
0.1	1,529,400	6,380	0.999
1	151,940	633	0.996
5	29,493	123	0.977
10	14,188	59	0.953
20	6,535	27	0.897
30	3,984	16	0.832

Determination of the water activity

Based on the fraction of free water in the hydrolysis calculated for high protein concentrations, a decrease in the water activity (a_w) is expected. However, despite the decrease in the free water, for all initial protein solutions the measured a_w values are close to that of water itself. The water activity is only decreasing from 1 to 0.997 with protein concentration increasing from 0.1 to 30 % (w/v). (**table 3**) For chemical reactions, the chemical potential of water, which is reflected by the water activity, would be important. The values determined would indicate that even at the highest concentrations of protein the water is not limiting. Still, based on the calculations it is expected that a significant amount of water would also be associated with the protein.

Table 3. Fraction of free water, free/bound water ratio and water activity, for different initial WPI concentrations before and after hydrolysis with Alcalase.

	Fraction free water			Free /bound water ratio			Water activity a_w			
Protein concentration (% (w/v))	initial	hydrolysates at 8000 s	% decrease	initial	hydrolysates at 8000 s	% decrease	initial	hydrolysates at 8000 s	Δa_w	DH reached
0.1	0.999	0.999	0.06	2,287	962	57	1.000	1.000	0.000	33.3
1	0.996	0.991	0.5	226	113	50	1.000	0.999	0.001	24.2
5	0.977	0.955	2.2	43	22	47	1.000	0.996	0.004	21.0
10	0.953	0.913	4.1	20	11	45	0.999	0.992	0.007	18.9
20	0.898	0.828	7.7	9	5	43	0.999	0.981	0.018	15.2
30	0.832	0.738	11	5	3	42	0.997	0.961	0.035	13.3

Determination of the fraction of free water by NMR

To quantify experimentally the fraction of bound and free water the relaxation rates of the water and protein in the solutions were determined by NMR. The peak corresponding to the free water has a relaxation time (T_2) of 2.4 s at 0.1 % (w/v) WPI. By increasing protein concentration, the relaxation rate of the free water decreases to 0.1 ms for 30 % (w/v) WPI. This indicates an increasing proportion of bound water with increasing protein concentration as described for cellulose at decreasing moisture content [32]. In parallel to this, the amplitude of the water signal is proportional to the protein concentration, indicating a complete recovery of the water signal. The relaxation rate ($1/T_2$) shows two regimes as a function of initial protein concentration. (**figure 4A**)

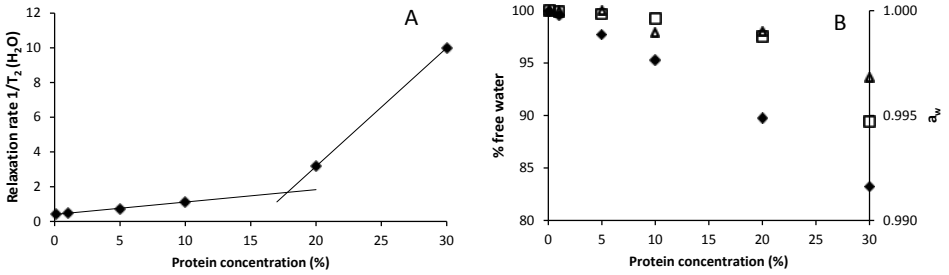

Figure 4. (A) Relaxation rate ($1/T_2(H_2O)$) of the water molecules and (B) % of free water as a function of protein concentration (0.1-30 % (w/v) WPI) (□) as measured by NMR and (♦) as calculated by equation (9) – (△) water activity (a_w) of the initial protein concentration as a function of protein concentration.

A first linear relationship is observed for 0.1-10 % (w/v) WPI. The relaxation rate varies from 0.4 for 0.1 % WPI to 1.1 ms^{-1} for 10 % (w/v) WPI. The second linear regime is observed for concentrations > 10 % (w/v) WPI for which, the relaxation rate reaches a value of 10 ms^{-1} at 30 % (w/v) WPI. Moreover, using the relaxation rate values and equation (13), it is determined that increasing the protein concentration from 0.1 % to 30 % (w/v) WPI, decreases the free water by 10 %. (**figure 4B**) This decrease shows the same trend as the fraction of free water calculated by equation (10) and as the a_w. Still, the calculations showed a decrease of almost 20 % and by NMR only 10 % decrease was measured.

Water availability during hydrolysis

Water activity

At the start of the hydrolysis of 0.1 % (w/v) WPI, the fraction of free water is 0.999, which corresponds to a ratio free/bound water of 2,287. (equation 11) For 30 % (w/v) WPI, the fraction of free water is 0.83 and the ratio free/bound water is 5. (**table 3**) The free/bound water ratio, which describes the excess of water, is significantly decreased by the increase in protein concentration. In addition, the ratio free/bound water also decreases during hydrolysis. After 8,000 s of hydrolysis, the ratio free/bound water is 962 for 0.1 % WPI and 3 for 30 % (w/v) WPI. (**table 3**) This translates into a decrease of 57 % of the available amount water at low concentrations and a decrease of 42 % at high protein concentrations after 8,000 s of hydrolysis at different DH. In these hydrolysates, the actual water activity was measured as an indication of the available water. (**table 3**) Although the values of the a_w of the hydrolysates decreases after hydrolysis, the decrease is small (≤ 0.035) for all samples. This difference in water activity (3 % decrease) is much smaller than expected based on the decrease in available water, determined from calculation of the free/bound water ratio (40 % decrease). It seems that the changes in water availability are not reflected by changes in the a_w as was also observed for the initial protein solutions. (**table 3**) The small effects of concentration and subsequent hydrolysis on the water activity have also been observed for hydrolysates of 10 % (w/w) solutions of ovalbumin, soy protein isolate (SPI) and casein hydrolyzed by trypsin, reaching values of a_w above 0.96 even at a DH of 50 % for SPI [33]. This shows that the water activity is a poor indication of the actual availability of water.

Water availability during hydrolysis

To investigate if the concentration of water plays a role during the hydrolysis, the free/bound water ratio was determined at each time point and related to the local rate of hydrolysis (dDH/dt) at that time point. The correlation between the local rate of hydrolysis and the free/bound water ratio provides an indication of the effect of water availability on the hydrolysis. (**figure 5A**) For this correlation, two regimes are distinguished. In the first regime (up to 1 % (w/v)) the curves for low protein concentrations show individual curves. For low protein concentrations, the local hydrolysis rate is slowly decreasing with the decrease in substrate concentration. The end of the hydrolysis is reached at different free/bound water ratios for the different substrate concentrations, 962 for 0.1 % (w/v) or 113 for 1 % WPI (w/v) hydrolyzed by Alcalase. In the second regime, for concentrations ≥ 5 % (w/v), the curves of different concentrations collapse onto one master curve. In this regime, the local hydrolysis rates decrease much faster with decreasing free/bound water ratio than in the first regime. Since enough substrate is available and the local rate of hydrolysis decreases at a similar ratio for all protein concentrations, this indicates that the water is the limitation in the hydrolysis.

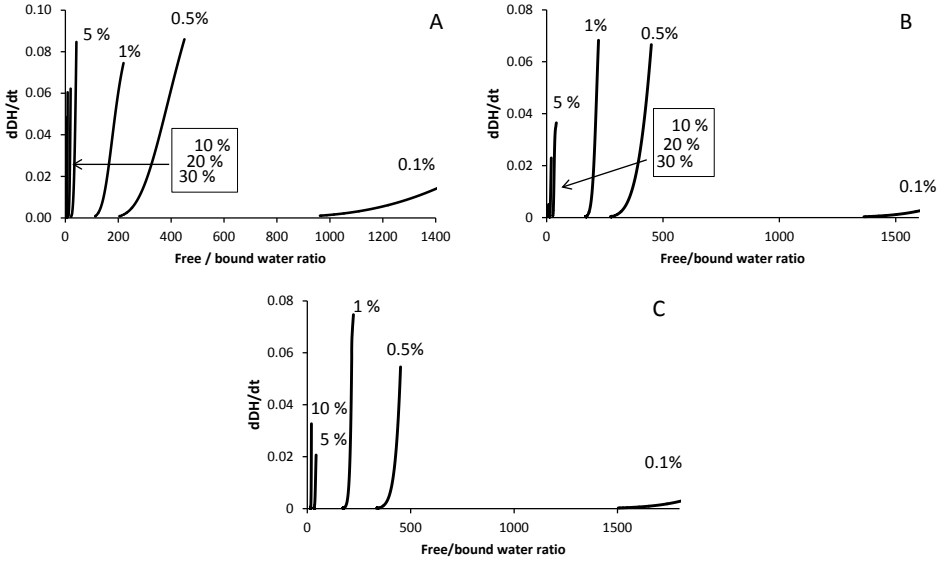

Figure 5. Local rate dDH/dt (Derivative of the DH vs time curve at each time point) as a function of the free/bound water ratio for 0.1-30 % (w/v) WPI hydrolyzed (A) by Alcalase; (B) by Neutrase and (C) by BLP.

These calculations were also performed for WPI hydrolyzed by Neutrase and BLP. (**figures 5B and 5C**) The observations are comparable to these with Alcalase, for the concentrations ≥ 5 % WPI. All curves collapse onto one curve, with a fast decrease in the rate of hydrolysis at a free/bound water ratio below 20. The results indicate that at free/bound water ratios below 20, the local rate of hydrolysis is independent of the substrate concentration, but dependent on the availability of the water. A similar observation has been made for the hydrolysis of β-casein [34]. It was shown in that study that while β-casein was hydrolyzed by chymosin at 1 % (w/v), no hydrolysis was detected at 50 % (w/v) casein [34]. It was defined [34] that there was 1.2 g water per g protein while the hydration of β-casein requires 2.1 g water per g protein. This corresponds to a free/bound water ratio of 2 for the initial solution (equation 11). It was concluded in that study that at this high protein concentration, the hydration of the molecules had priority over the use of water for the hydrolysis. This confirms our observations that below a ratio of free/bound water of 20 the hydrolysis rate depends on the availability of water and in that case, not enough water was available for the hydrolysis to start.

Addition of co-solutes to the protein solutions

If the available amount of water is indeed limiting the hydrolysis, this effect should also be observed when other (non-protein) co-solutes are added to the protein solution. As a reference, high concentrations of co-solutes were added to 1 and 10 % (w/v) WPI solutions and compared to solutions with high protein content. The addition to a final

concentration of 29 % (1.6 M) glucose to a 1 % (w/v) WPI solution decreases the free/bound ratio water from 226 to 7. The presence of 20 % glucose (1.1 M) in a 10 % WPI solution reduces the free/bound water from 20 to 6. (**table 4**)

Table 4. Initial free/bound water ratio and water activity (a_w) for WPI solutions with co-solutes.

Protein concentration (% (w/v))	Co-solute	Free /bound water ratio	a_w (initial solution)
1	29 % glucose	7	0.973
10	20 % glucose	6	0.980
1	1 M proline	16	0.986
1	0.5 M NaCl	21	0.987
1	6 M NaCl	0.8	0.754
10	6 M NaCl	0.5	0.754
30	6 M NaCl	0.1	0.752

The addition of glucose to the low protein concentrations, besides increasing the total mass in the solution, allows the characterization of the influence of osmolarity in the solutions. The presence of 29 and 20 % (w/v) glucose is equivalent to an osmolarity of 1.6 or 1.1 osmol·L^{-1}, respectively. This is much higher than the osmolarity of the 30 % (w/v) WPI (11.10^{-3} osmol·L^{-1}). The a_w of the protein solutions in the presence of glucose is decreased to 0.973 and 0.980, respectively, which corresponds to the water activity of the glucose solution alone [35]. The effect on the a_w is higher for the addition of glucose than the effect of protein concentration. Still, the free/bound water ratio is decreasing by 60-95 % while the a_w is only decreasing by 2-3 %. The addition of glucose decreased the initial rate of hydrolysis by Alcalase compared to the pure WPI solutions. The 10 % WPI + 20 % glucose has a similar initial rate compared to 30 % WPI. Furthermore, the presence of glucose did not affect the final DH. (**figures 6 and 7**) Hence, the lower DH reached at higher protein concentration is not the result of increasing osmolarity. A comparable conclusion was reached for the enzymatic conversion of cellulose, in which the replacement of water by an inert oil (oleyl alcohol) had a smaller effect than the increase in substrate concentration [10].

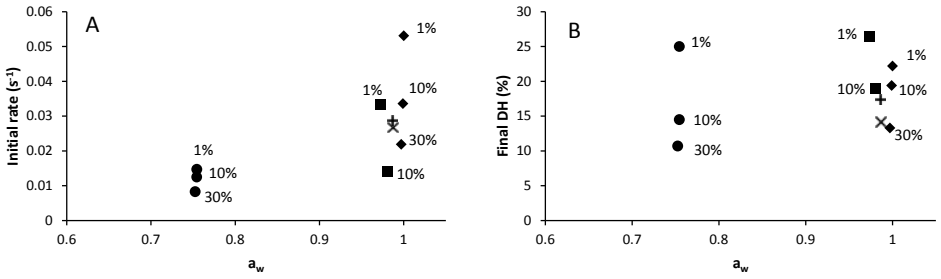

Figure 6. Influence of the a_w on (A) the initial rate and (B) the final DH for (♦) WPI solutions at concentration 1, 10 and 30 % (w/v); (■) 1 and 10 % (w/v) WPI with 29 % and 20 % glucose, respectively; (●) 1, 10 and 30 % (w/v) WPI with 6 M NaCl solutions; (**x**) 1 % WPI + 0.5 M NaCl and (**+**) 1 % WPI + 1 M proline, hydrolyzed by Alcalase at constant enzyme/substrate.

To confirm the role of water availability on the hydrolysis, NaCl was added to a final concentration of 0.5 M to reach a free/bound water ratio of 21. The addition of NaCl only decreases the a_w to 0.987. (**table 4**) The initial rate of hydrolysis and final DH for the 1 % WPI + 0.5 M NaCl are decreased compared to that of the 1 % WPI solution. This supports the hypothesis that the initial rate of hydrolysis and DH reached are controlled by the water available described here by the free/bound water ratio.

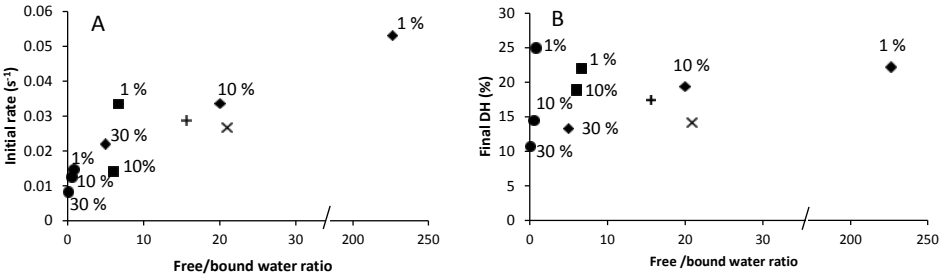

Figure 7. Influence of the initial free/bound water ratio on (A) the initial rate of hydrolysis and (B) on the final DH for (♦) WPI solutions at concentration 1, 10 and 30 % (w/v); (■) 1 and 10 % (w/v) WPI with 29 % and 20 % glucose respectively; (●) 1, 10 and 30 % (w/v) WPI with 6 M NaCl solutions, (**x**) 1 % WPI + 0.5 M NaCl and (**+**) 1 % WPI + 1 M proline, hydrolyzed by Alcalase at constant enzyme/substrate.

An additional experiment was performed by addition of proline to a final concentration of 1 M to a 1 % WPI solution. In these conditions, a free/bound water ratio of 16 is reached, slightly lower than for 1 % WPI + 0.5 M NaCl, but comparable a_w (0.98) to a_w of 1 % WPI + 0.5 M NaCl. The hydrolysis of 1 % WPI + 1 M proline leads to comparable final DH and initial rate of hydrolysis to the one obtained at 1 % WPI + 0.5 M NaCl and lower than 1 % WPI alone. Hence, the decrease of available water results in a decrease in the initial rate of hydrolysis caused by addition of protein, amino acid or NaCl. The hydrolysis in the presence of proline and NaCl are comparable, indicating that there is no specific effect of this amino acid.

In the presence of co-solutes lower final DH values are obtained than for the protein solutions alone. At the same time, even in the presence of co-solutes a lower DH is reached at 10 % than at 1 % (w/v) WPI. It has been described that at increasing concentrations of N-acetyl amino acid amide the hydration number (i.e. mole of water molecules per mole of solute) decreased [36]. This indicates that at increasing protein concentration, the hydration of the amino acid residues is different than at low protein concentrations. This might also indicate that the hydration is different in the initial solution than at high DH. The lower hydration number of the protein at increasing protein concentration might be the reason for a different accessibility of the substrate by the enzyme. A lower accessibility of the substrate might be the reason for a lower DH obtained at increasing protein concentration.

To change the free/bound water ratio more dramatically, additional experiments were performed after addition of 6 M NaCl to 1, 10 and 30 % (w/v) protein solutions. It has been described that the mean activity coefficient of NaCl in water is decreasing to 0.65 with increasing NaCl concentrations for concentrations up to 1 M NaCl. Above this concentration and up to saturation of the solution, the mean activity coefficient is increasing back to 1. So, the mean activity coefficient at 0.5 M NaCl is 0.68 and at 6 M NaCl it is 0.98 [37]. This gives an indication that at concentrations as high as 6 M NaCl, the NaCl forms a system by itself and its behavior is close to ideality. Still, the addition of NaCl to a final concentration of 6 M increases the number of charges present in the solution and thus decreases the free/bound water ratio. Consequently, at all protein concentrations free/bound water ratios below 1 are obtained: 0.8, 0.5 and 0.1 for 1, 10 and 30 % WPI, respectively, in the presence of 6 M NaCl. In addition, in all cases the a_w is decreased to 0.75. (**table 4**) At this high NaCl concentration, the contribution of the proteins is small compared to that of NaCl; the a_w of a 6 M NaCl solution is 0.75. Compared to the WPI solutions, a decrease of the free/bound water ratio of 97-99 % is calculated, but a decrease of only 25 % of the a_w is measured. At these extreme values, a lower initial rate is observed for the different protein concentrations compared to the pure protein solutions. The DH reached are, however, not significantly affected by the high NaCl concentration. (**figures 6 and 7**) Moreover, the effect of substrate concentration on the enzymatic hydrolysis is not changed, with a higher DH reached at low substrate concentration than at high substrate concentrations. (**figures 6 and 7**) This shows that the water availability plays a role in the hydrolysis and in particular in the initial rate of hydrolysis. But in parallel, the effect of increasing protein concentration on the final DH might be the result of a secondary effect besides water availability.

Conclusions

The increase of the protein concentration or the addition of co-solutes reduces the amount of available water as determined by the free to bound water ratio. The changes in ratio are, however, not reflected by the measured water activity. Both the increase in protein concentration and the addition of co-solutes lead to a decrease in the initial free to bound water ratio of the solutions. The decrease in free to bound water ratio correlates with a lower initial rate of hydrolysis and final DH.

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Chapter 4

Introducing enzyme selectivity: A quantitative parameter to describe enzyme preference within a given specificity

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Abstract

In this study, the selectivity of a protease is introduced as a quantitative parameter that describes the relative rate at which each possible cleavage site based on a given specificity, is hydrolyzed compared to others. A method was developed to quantify all the peptides formed during hydrolysis in terms of molar concentration, based on the UV_{214} signal. *Bacillus licheniformis* protease (BLP) was used, since it is highly specific for Glu and Asp residues. A whey protein isolate was used, that contains β-lactoglobulin (genetic variants A and B) and α-lactalbumin. For each hydrolysate, taken at different DH, at least 90 % of the total UV_{214} peak area was included in the analysis. The quality of identification and quantification of the peptides were described by newly defined parameters: peptide sequence coverage and molar sequence coverage. The peptide sequence coverage is an indication of the fraction of annotated amino acids based on the total number of amino acids in annotated and missing peptides. The molar sequence coverage indicates the ratio at which all the amino acids of the sequence of the parental protein were quantified, in relation to the original concentration of protein. A total of 58 peptides resulting from the hydrolysis of β-lactoglobulin were annotated and quantified, resulting in an average peptide sequence coverage of 94 % and an average molar sequence coverage of 75 %. The increase of cleavage products from each cleavage site as a function of time was used to determine the rate of hydrolysis of that cleavage site. The selectivity was calculated as the relative rate of hydrolysis of the different cleavage sites. Clear differences in terms of enzyme selectivity towards the 16 glutamic acid cleavage sites in β-lactoglobulin were observed. Five groups were identified (4 Glu with ± 16 %, 5 with ± 6 %, 4 with ± 1.4 %, 1 with 0.03 % and 2 with 0 % selectivity), thereby elucidating the selectivity of the enzyme. This makes selectivity an essential parameter to increase the understanding of interactions between enzyme and substrate as well as the effect of system conditions.

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Introduction

Description of the hydrolysis

While enzymatic hydrolysis of proteins is quite common, detailed understanding of the hydrolysis process is still lacking. This is partly due to the absence of methods to describe quantitatively the complete assortment of peptides formed during the hydrolysis process. One commonly used parameter to describe the hydrolysis is the degree of hydrolysis [1]. This is a global parameter, which does not provide information on the mechanism of hydrolysis. It solely describes the proportion of hydrolyzed peptide bond, and provides no information about which bonds were hydrolyzed. To provide a more detailed description of the activity of the enzyme, and thereby the mechanism of hydrolysis, the terms specificity and preference (or selectivity) are used in literature. Although sometimes used interchangeably the consensus seems to be that the *specificity* of the enzyme describes the type of amino acid after which the enzyme can hydrolyze a peptide bond, (e.g. Lys and Arg for trypsin). The latter is also referred to as the cleavage site. Not all cleavage sites are hydrolyzed at the same rate [2,3]. The rate of hydrolysis of a specific cleavage site is affected by the presence of other amino acids (subsite) in the positions close to the cleavage site [4,5]. This has been qualitatively described by the term preference. However, as will be discussed later, other factors may also result in faster hydrolysis of one cleavage site compared to another. We, therefore, propose to define the term *selectivity* as a quantitative parameter that describes the relative rate at which each individual cleavage site is hydrolyzed within a given specificity. In other words, the selectivity is the rate of hydrolysis of one peptide bond compared to the total rate for the enzyme at which all peptides bonds after the same type(s) of amino acid(s) at different positions in the protein are hydrolyzed.

Factors influencing the selectivity

The above defined selectivity of enzyme towards specific cleavage sites may be influenced by four different factors. A first factor is the charge state of the (different) amino acids in the substrate, for enzymes that are specific for two or more charged amino acids, such as trypsin. An example is the effect of pH (7-10) on the release of peptides during trypsin hydrolysis of β-casein [6]. With increasing pH, the rate of hydrolysis is decreased for Lys, while that of Arg is constant. Consequently, the selectivity for Lys is decreased, while that for Arg is increased. This seems to correlate with the charge of the amino acid side chains. The pKa of Lys (10.5) is lower than that of Arg (12.4). Secondly, changes in temperature can result in differences in the selectivity. This has been suggested for a glutamyl endopeptidase (*Bacillus licheniformis* protease) by comparing qualitatively the hydrolysis of β-casein [5,7]. Different peptides were obtained at two temperatures after a similar time of hydrolysis. Some bonds after Glu residues were cleaved preferentially at 37 °C, whereas others were preferred at 50 °C. Thirdly, the role of the subsite has been mentioned. This refers to the interaction between the amino acids that form the catalytic site of the protease and how they affect the interaction with the amino acids surrounding the cleavage site

on the substrate [8]. The hydrolysis after glutamic acid residues was, for example, hindered by the presence of Met or Pro at position P1' [5]. Finally, selectivity may also be affected by the accessibility of the substrate, i.e. by the folding state and aggregation of the protein substrate. It is generally observed that the kinetics of hydrolysis is faster for unfolded proteins and aggregated proteins than for proteins in their folded state [9,10]. However, it is not clear how such changes affect the peptide formation.

Monitoring the hydrolysis

The changes in enzyme selectivity result in changes in the hydrolysis mechanism, i.e. which peptides are formed and when. To be able to identify these changes, a quantitative description of the formation (and subsequent breakdown) of individual peptides during the hydrolysis is needed. This is sometimes referred to as peptide release kinetics [11]. In the past years several articles have been published that describe the course of peptide formation as a function of time using semi-quantitative techniques. For instance, the peak areas of MS extracted ions (extracted current ion) were used to monitor the peptides formed [12]. With this method the relative abundance of individual peptides was monitored as a function of the DH. Still, it did not allow comparison of the abundance between peptides in terms of absolute concentration due to differences in ionization efficiency of different peptides. To correct for this, isotopically labeled standards for each individual peptide would be needed, making it an unrealistic approach [13]. Moreover, the MS based quantification implies that the intensity is proportional to the concentration. However, the intensity can be affected by ion suppression, the presence of sodium adducts, or by several m/z peaks for one compound. To avoid these problems, a (label-free) absolute quantitative method has previously been developed using the RP-UHPLC-UV signal, taking into consideration the geometry of the UV detector [14]. This method was based on earlier semi-quantification analysis of peptides using the calculated extinction coefficient at 214 nm of each peptide [15]. It has since then been used, among others, to determine the exact molar concentration of several peptides formed during the hydrolysis of casein by *Lactococcus lactis* [14], for the quantification of peptides in the total hydrolysate of β-lactoglobulin obtained after hydrolysis by BLP [16], and for β-lactoglobulin hydrolyzed by trypsin [11].

Approach

From the above it is clear that currently used descriptors of hydrolysis (e.g. DH, specificity) do not provide sufficient insight in the hydrolysis mechanism. Over the past decade there have been developments in methodology and absolute quantification of peptides. Nevertheless, there is still a lack of definitions and methods to describe the hydrolysis process in a quantitative manner. In this paper, methods were developed for the annotation and quantification of peptides in hydrolysates obtained at different DH during hydrolysis of 1 % (w/v) WPI by BLP. To ensure and describe the quality of

analysis, a new set of parameters is defined. Based on the peptide quantification, enzyme selectivity is introduced as a quantitative parameter to describe the relative rate at which a cleavage site is hydrolyzed.

Materials and Methods

Materials. Bipro, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). It contained (by weight) 74.0 % β-lactoglobulin, 12.5 % α-lactalbumin, 5.5 % bovine serum albumin and 5.5 % immunoglobulin. Based on this composition, the proteins present can be represented as a theoretical protein of 239 amino acids with 22 Glu and 17 Asp residues present. (chapter 3) The protein content (Nx6.32) [17] of the powder was 93.4 % (w/w) as determined by Dumas. BLP (*Bacillus licheniformis* protease), specific for Glu-X bonds and for Asp-X bonds [18], was obtained from Novozymes (Bagsvaerd, Denmark). BLP (NS-37005) had an activity of 0.3 AU/mg/min as determined by azocasein assay [19]. BLP (4.5 % (w/w) protein, Nx6.25) was partly insoluble and was fractionated as described previously (chapter 3). The resulting soluble fraction was freeze dried. The freeze dried material was found to contain 60 % (w/w) protein (Nx6.25) and an activity of 3.9 AU/mg/min was determined by azocasein assay. The resulting material was found to contain 78 % of BLP (23.6 kDa) and 14 % of propeptide (6.9 kDa) as determined previously (chapter 3). All other chemicals were of analytical grade and purchased from Sigma or Merck.

Hydrolysis

A 1 % (w/v) protein solution was prepared by dispersing WPI powder at a concentration of 45 % (w/v), followed by stirring overnight at 4 °C. Insoluble parts were removed by centrifugation (30 min, 4000 g, 20 °C) and the supernatant was diluted to 1 % (w/v) based on UV_{280} as described before [17]. The 1 % (w/v) WPI solution was hydrolyzed at 40 °C and pH 8.0 using 0.2 M NaOH to keep the pH constant using a pH-stat. The protein solution (10 mL) was preheated 15 minutes at 40 °C and adjusted to pH 8.0 before addition of BLP dissolved at 5 % (w/v) in Millipore water (0.30 µL of enzyme/ mg of protein). The degree of hydrolysis (DH) was calculated based on the volume of NaOH added using $1/\alpha = 1.20$ at 40 °C and pH 8.0 [20] and h_{tot}(WPI) = 8.5, the total number of peptide bonds per gram protein substrate, as described before [17]. Samples were taken during the hydrolysis at different degrees of hydrolysis (1.5, 3, 4.5, 6 and 7 %). The samples were directly centrifuged (5 min, 19000 g, 15 °C), yielding a supernatant and pellet which were separated. The pellet was re-dispersed in the original volume using millipore water and all supernatants and pellets were inactivated by adjusting the pH to 2 with 5 M HCl. The pH was set back to 8.0 after at least 10 minutes of inactivation before storage of the samples at – 20 °C.

Solubility

The protein contents in the supernatants of the samples taken at different DH values were determined using DUMAS (Nx6.32) [17] using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The proportion of soluble protein was calculated by dividing the concentration of protein of the supernatants by the concentration of protein in the solution before centrifugation at DH=0.

Reverse Phase Ultra High Performance liquid chromatography (RP-UHPLC)

Samples obtained during hydrolysis were analyzed on an Acquity UPLC System (Waters, Milford, MA, USA) using an Acquity UPLC BEH 300 C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 x 50 mm, 1.7 µm particle size). Eluent A was 1 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) in Millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. To reduce disulfide bridges and to facilitate peptide annotation, samples were first incubated for two hours with 100 mM dithiothreitol (DTT) in 50 mM Tris·HCl buffer pH 8.0 at a concentration of 0.5 % (w/v). After incubation, samples were further diluted in eluent A to a final concentration of 0.1% (w/v) and centrifuged (10 min, 19000 g, 20 °C). Supernatants (4 µL) were injected into the column thermostated at 40 °C.

The amount of remaining intact protein was determined using the following elution profile: 0-2 min isocratic on 30 % B; 2-12 min linear gradient from 30 % B to 50 % B; 12-15 min linear gradient from 50 to 100 % B; 15-20 min isocratic on 100 % B; 20-21 min from 100 % B to 30 % B and 21-30 min isocratic on 30 % B. The proportions of remaining intact α-lactalbumin and β-lactoglobulin were determined by dividing the area of the UV_{214} peak obtained for each protein at different DH by the peak area obtained for each intact protein before hydrolysis.

A second elution profile was used for separation of the peptides: 0-2 min isocratic on 3 % B; 2-10 min linear gradient from 3 % to 22 % B; 10-16 min linear gradient 22-30 % B; 16-19 min linear gradient 30-100 % B; 19-24 min isocratic on 100 % B; 24-26 min linear gradient 100-3 % B and 26-30 min isocratic on 3 % B. The flow rate was 350 $\mu L \cdot min^{-1}$. Detection was performed using a PDA, which scanned the absorbance from 200-400 nm at a 1.2 nm resolution, with 20 spectra per second.

Electron spray ionization time of flight mass spectrometry (ESI-Q-TOF-MS)

The mass spectra of the hydrolysates were determined with an online Synapt high definition mass spectrometer (Waters), coupled to the RP-UPLC system, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). The system was calibrated using Glu-1-Fib. The capillary voltage was set to 3 kV with the source operation in positive ion mode and the source temperature at 120 °C. The sample cone was operated at 35 V. Nitrogen was used as desolvation gas (250 °C, 800 L/h) and cone gas (200 L/h). The trap gas was set at 1.5

mL/min. MS and MS/MS (MSe method) were performed between m/z 100-2000 with a scan time of 0.3 seconds. The trap collision energy was set at 6 V in single MS mode and ramped from 20 to 30 V in MSe mode. The transfer collision energy was set at 4 V in MS and switched between 4 and 10 V in the MSe mode. UV and MS data were acquired using MassLynx software v4.1 (Waters).

Peptide identification and quantification

Internal lock mass was applied on every chromatogram for MS and MS/MS data with two previously identified masses chosen among the most abundant masses, of which the sequences were identified manually. The detection of MS and MSe ion peaks by Biopharmalynx 1.3 software (Waters) was set to an intensity limit of 10 counts. For the annotation, a mass tolerance of 0.1 Da was set for both MS and MSe data. In the final analysis, only peptides annotated with b and y fragments were used. Peptides were annotated with Biopharmalynx software, using first a method to identify peptides cleaved after Glu and Asp residues. Next, a generic method was used in which no enzyme specificity was selected. Peptides from both variants of β-lactoglobulin (A and B) as well as peptides from α-lactalbumin were annotated. Peptides that were annotated in both methods were considered to be correctly identified. Those peptides that were annotated in only one of the methods were verified manually. Each annotated peptide was subsequently quantified using the UV_{214} peak area from the corresponding retention time [16]. For co-eluting peptides, the corresponding UV_{214} peak area was distributed over the two peptides based on their relative abundance in MS. This is based on the assumption that co-eluting peptides have similar ionization, since they should have comparable overall properties (i.e. charge, size and hydrophobicity). For UV peaks where the automatic annotation did not yield a peptide, a manual analysis of the MS and MS/MS data was used to assign the peptide.

The sensitivity, which is the ability to identify the peptides (i.e. ability to avoid false negatives) was defined as:

(1) $$sensitivity = \frac{number\ of\ peptides\ correctly\ identified}{total\ number\ of\ peptides\ giving\ rise\ to\ significant\ MS\ signal}$$

By using Biopharmalynx software a sensitivity of 45 % (± 5%) is obtained, which was improved to 75 % (± 5%) after manual identification. The specificity is the ability to avoid false positives and is defined as:

(2) $$specificity = \frac{number\ of\ peptides\ correctly\ identified}{total\ number\ of\ peptides\ identified}$$

The specificity of the identification was 60 % (± 10 %). For the total peptide quantification peaks in the UV_{214} chromatogram were included until 90-95 % of the total UV_{214}. Peptides from both β-lactoglobulin and α-lactalbumin were included. These included peptides from both A and B variants of β-lactoglobulin, which were found to be present in Bipro as 60 and 40 % of the total β-lactoglobulin, as determined by RP-UPLC [21]. 58 different peptides derived from β-lactoglobulin and 10 peptides derived from α-lactalbumin were identified. Only peptides from β-lactoglobulin were used to determine the selectivity of the enzyme.

The quantification of the peptides was based on the UV signal at 214 nm, using equation (3):

(3) $$C_{peptide} = 1 * 10^6 \left(\frac{A_{214}}{\varepsilon_{214} l V_{inj} k_{cell}}\right) Q$$

In which $C_{peptide}$ (µM) is the concentration of peptide, A_{214} (AU.min) is the UV peak area at 214 nm, V_{inj} (µL) is the volume of sample injected, Q the flow rate in µL·min^{-1}, l is the path length of the UV cell which is 1 cm according to the manufacturer. The value for the cell constant k_{cell} was previously determined to be 0.66 using pure peptides, but depends on the geometry of the UV detector used [16]. The molar extinction coefficient at 214 nm of each peptide (ε_{214}) was calculated as described before [22].

Reproducibility of annotation and quantification

To test the reproducibility of the quantification method, one sample (1 % WPI, DH 4.5 %) was injected 3 times. In addition, the reproducibility of the complete analysis was tested. For this, two separate hydrolysis experiments were performed at the same protein concentration (0.5 % (w/v) WPI). During each hydrolysis, samples were taken at DH 1.5, 3, and 6 %. Peptides were annotated and quantified for all hydrolysates. The error was calculated for the hydrolysates at each DH. The values of errors were averaged to determine the analysis error. The reproducibility of the annotation, quantification and determination of the selectivity was obtained from two sets of hydrolysates obtained from two separate experiments. The quality of the annotation as described by the peptide sequence coverage showed a standard error of 2.5 %. The molar sequence coverage showed a standard error of 18 %. Finally, the determined selectivity showed a typical standard error of 15 %. This shows a good reproducibility of the analysis and of the selectivity obtained from two separate experiments.

Results and discussion

Description of the hydrolysates

The degree of hydrolysis (DH) reached a value of ± 8 % after 10,000 s of hydrolysis of 1 % (w/v) WPI by BLP. (**figure 1A**) Considering hydrolysis taking place after Glu residues, a maximal DH of 9.5 % was expected for this WPI. If Asp residues are included a maximal DH of 16 % can be reached. The DH value obtained after 10,000 s shows that not all cleavage sites of WPI have been hydrolyzed or not to the full extent.

The decrease of the amount of intact protein as a function of the DH gives an indication of the preference for the hydrolysis of intact proteins versus the hydrolysis of intermediate peptides formed and thereby the selectivity. For the hydrolysis of 1 % WPI by BLP, there is no remaining intact β-lactoglobulin above DH 4.5 % and still 40 % remaining intact α-lactalbumin at DH 4.5 %. (**figure 1B**)

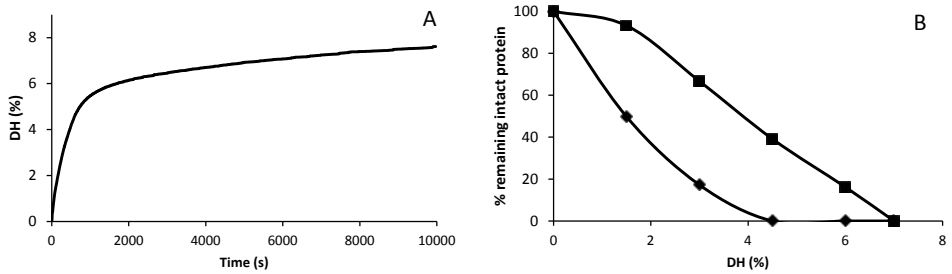

Figure 1. (A) Hydrolysis curve and (B) remaining (♦) intact β-lactoglobulin (A + B) and (■) intact α-lactalbumin as a function of DH determined by the pH-stat, for the hydrolysis of 1 % (w/v) WPI by BLP (pH 8 - 40°C).

The enzyme hydrolyzes preferentially β-lactoglobulin compared to the peptides and compared to intact α-lactalbumin. The preference of the enzyme for β-lactoglobulin compared to α-lactalbumin is expected, based on the higher amount of β-lactoglobulin present in WPI. In addition, β-lactoglobulin contains relatively more Glu residues than α-lactalbumin. Before the hydrolysates were analyzed, the solubility was determined to ensure that all peptides were included in the analysis. The solubility of the hydrolysates is on average 95 ± 2 %. Hence, the pellet was not analyzed further. The high solubility shows that there is no formation of insoluble aggregates, which might affect the rate of hydrolysis. It also indicates that the peptides identified represent the complete sample. Hence, the hydrolysates can thus be compared for detailed analysis of the hydrolysates and to determine the selectivity of the enzyme.

Identification of the peptides in the hydrolysates

To describe the mechanism of hydrolysis via the selectivity of the enzyme, the absolute amounts of peptides present, in terms of molar concentration, formed during hydrolysis need to be determined. The first step in this process is the correct annotation of the peptides analyzed in the LC-MS chromatograms. Peptides from β-lactoglobulin and α-lactalbumin were annotated. A typical RP-UHPLC-UV_{214} chromatogram of an hydrolysate (1 % WPI – DH 7 %) is shown in **figure 2A**.

To illustrate the procedure for peptide identification, the mass spectrum of the peak at 11.9 min is displayed (**figure 2B**) as well as the corresponding MS/MS spectrum. (**figure 2C**) The molecular mass of the peptide eluting at this retention time is 928.54 Da. A database search indicated that the peptide is fragment β-lg[56-62]. The annotation was further confirmed by identification of b and y fragments by MS/MS. (**figure 2C**) Using this approach a total of 58 different peptides derived from β-lactoglobulin and 10 peptides derived from α-lactalbumin were annotated in the hydrolysates obtained at different DH values. The accuracy of the identification was verified by calculating the error on each mass, which was found to be on average ± 10 ppm. (**table 1**) In the next sections the different parameters used to determine the quality of the analysis are discussed.

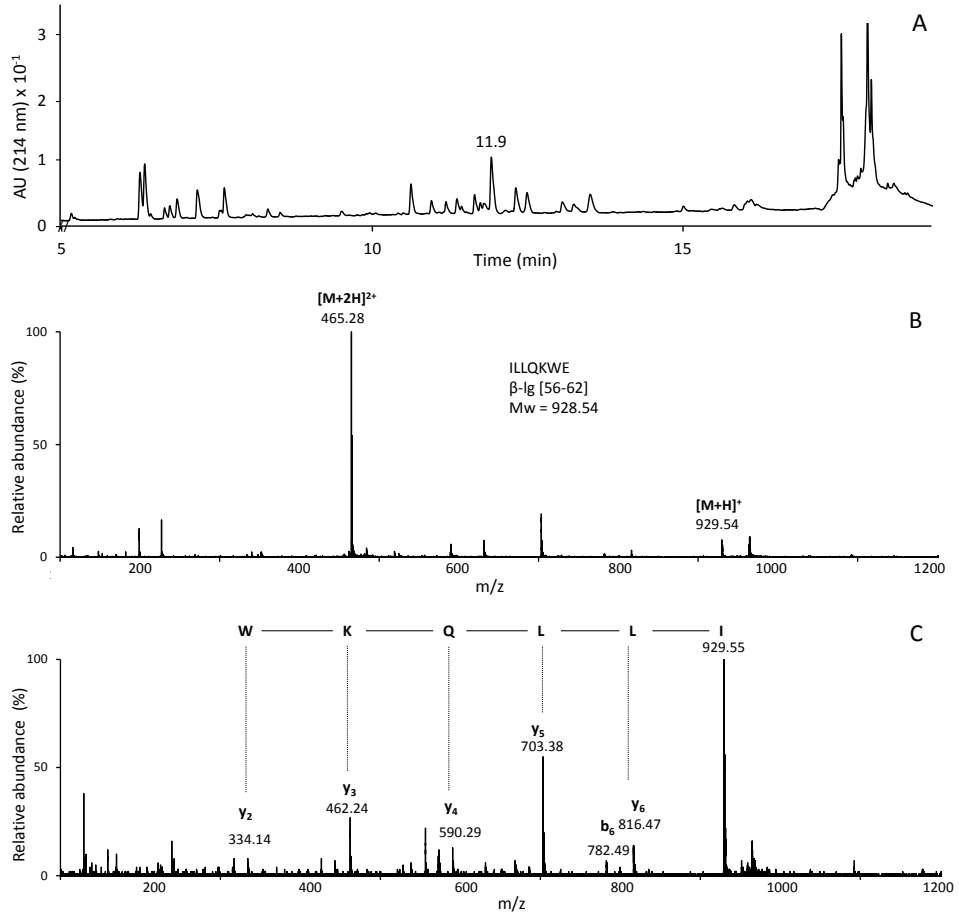

Figure 2. Procedure of annotation of peptide β-lg[56-62] with (A) UV_{214} chromatogram for 1 % (w/v) WPI at DH 7 %. (B) MS scan of the peak at 11.9 min. (C) MS/MS of the peak at 11.9 min, annotation of the b and y fragments.

Qualitative sequence coverage

The quality, or completeness, of the annotation of peptides in an hydrolysate is typically described (e.g. in proteomics studies) by the sequence coverage. This value is calculated from the number (#) of unique annotated amino acids (i.e. for β-lg: Leu-1, Ile-2… Ile-162) divided by the total number (#) of amino acids in the protein sequence [23]. (equation 4) We will refer to this parameter as the amino acid sequence coverage.

(4) $$amino\ acid\ sequence\ coverage = \frac{\#\ unique\ annotated\ amino\ acids}{\#\ amino\ acids\ in\ protein\ sequence}$$

For all samples in this study all amino acids present in the parental protein were found at least once, yielding amino acid sequence coverages of 100 % both for α-lactalbumin and β-lactoglobulin. Still, this parameter is only a poor indication of the quality of the analysis. It does not indicate whether all peptides present are annotated.

Table 1. List of peptides derived from β-lactoglobulin identified in all hydrolysates of 1 % (w/v) WPI hydrolyzed by BLP. (* indicates a-specific peptides)

fragment	sequence	theoretical mass (Da)	mass observed (Da)	Error (ppm)	charge state	b fragments identified	y fragments identified
[1-11]	LIVTQTMKGLD	1217.669	1217.686	14	2+	2,5	4,5,6,7,8,9
[1-28]	LIVTQTMKGLDIQKVAGTWYSLAMAASD	3010.550	3010.536	-5	2+	4,10	
[1-33]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLD	3551.862	3551.883	6	3+, 2+	2,7	1,7,8
[1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	4894.550	4894.548	1	3+, 4+	2	1,4,7,8,14,23(2+)
[1-51]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPE	5559.925	5560.012	16	4+,	1,2,3,4,12	1,2,7,21(2+),22(2+),26(2+)
[1-55]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE LKPTPEGDLE	5974.099	5974.048	-9	3+,4+	1,2,21(2+),23(2+)	1,3,14(2+),18(2+),21(2+),24(2+)
[1-62]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE LKPTPEGDLEILLQKWE	6884.628	6885.172	79	4+	1,2	5,30(2+)
[29-45]	(D)-ISLLDAQSAPLRVYVEE	1902.009	1902.022	7	3+,4+	2,5,7,8,9,11,12,13,14,15,16	2,3,5,6,8,10,17
[34-45]	(D)-AQSAPLRVYVEE	1360.698	1360.714	12	2+,1+	2,3,8,9,10,11	1,6,8,9,10,12
[46-51]	(E)-LKPTPE	683.385	683.395	15	2+,1+	5	2,3,4,5,6
[46-55]	(E)-LKPTPEGDLE	1097.560	1097.576	15	2+,1+	2,4,6,8,9	1,6,7,8,9,10
[52-55]	(E)-GDLE	432.186	432.191	12	1+	3,4	1
[56-59]	(E)-ILLQ*	485.321	485.339	37	1+	2,3	1,2
[56-62]	(E)-ILLQKWE	928.538	928.548	11	2+,1+	5,6	2,3,4,5,6,7
[56-74]A	(E)-ILLQKWENDECAQKKIIAE	2271.193	2271.183	-4	3+,2+	2,5	2,5,6,9
[60-62]	(Q)-KWE*	461.227	461.233	13	1+	1,2,3	2
[63-74]A	(E)-NDECAQKKIIAE	1360.666	1360.676	7	2+,1+	2,3,4,5,6,8,9,10	1,2,3,4,5,7,8,9,10,12
[63-74]B	(E)-NGECAQKKIIAE	1302.660	1302.664	3	2+,1+	2,3,8,9,10	1,2,6,7,8,9,12
[63-89]A	(E)-NDECAQKKIIAEKTKIPAVFKIDALNE	3028.627	3028.644	6	3+,2+	1,2,3	11,15(2+),27(2+)
[63-89]B	(E)-NGECAQKKIIAEKTKIPAVFKIDALNE	2970.621	2970.624	1	4+,3+,2+	1,11	4,7,11,15(2+),24(2+),27(2+)
[63-108]A	(E)-NDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCME	5344.816	5344.828	2	4+,3+		2,3,4,16(2+),17(2+),19(2+)
[66-74]	(E)-CAQKKIIAE	1002.553	1002.560	7	2+,1+	1,2,3,5,6,7,8,	1,2,5,6,7
[66-89]	(E)-CAQKKIIAEKTKIPAVFKIDALNE	2670.514	2670.489	-9	3+,	13(2+),16(2+)	1,2,3,5,6,7,8,9,10,11,12,13,15
[75-80]	(E)-KTKIPA*	656.422	656.425	5	1+	1,2,3,4,6	2,5
[75-85]	(E)-KTKIPAVFKID	1258.765	1258.774	7	2+	1,2,3,4,7,8,9	1,7,8
[75-89]	(E)-KTKIPAVFKIDALNE	1685.972	1685.972	0	3+,2+,1+	1,2,3,4,6,7,8,11	7,8,11
[75-108]	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCME	4002.161	4002.120	-10	4+,3+	2,7	
[75-134]B	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLAC QCLVRTPEVDDEALE	6829.425	6829.480	8	4+		2,5,6,9
[81-89]	(A)-VFKIDALNE*	1047.560	1047.584	23	2+,1+	5,6,7,8	5,6,7,8,9

fragment	sequence	theoretical mass (Da)	mass observed (Da)	Error (ppm)	charge state	b fragments identified	y fragments identified
[81-89]	(A)-VFKIDALNE*	1047.560	1047.584	23	2+,1+	5,6,7,8	5,6,7,8,9
[86-89]	(D)-ALNE	445.217	445.226	20	1+	2,3	1,2,4
[90-108]	(E)-NKVLVLDTDYKKYLLFCME	2334.200	2334.198	-1	3+,2+	2,3,4,5,7,8,9,14(2+),15(2+), 16(2+),17(2+),18(2+)	1,2,3,4,15(2+),16(2+),17(2+)
[90-127]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPE	4418.167	4418.214	11	3+,4+	2,3,4,5,6	1,2,3,4,5,7
[90-127]B	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPE	4390.136	4390.179	10	3+,4+	2,3,4,5,6	4,5,25(3+)
[90-131]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDE	4876.332	4876.386	11	3+,4+	5,6,36(3+)	1,3,6
[90-134]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALE	5189.495	5189.520	5	3+,4+	1	1,2,6,9
[97-108]	(D)-TDYKKYLLFCME	1552.730	1552.746	10	2+	4,8,9	2,3,4,5,6,8
[109-127]A	(E)-NSAEPEQSLVCQCLVRTPE	2101.977	2101.988	5	2+,3+	2,3,4,6,7,8,9,12,13,14,15, 16,17	2,3,4,5,6,7,8,9,10,11,15
[109-127]B	(E)-NSAEPEQSLACQCLVRTPE	2073.946	2073.952	3	2+,3+	4,13,14,15,16	2,4,5,7,11,12,13,14,15
[109-131]A	(E)-NSAEPEQSLVCQCLVRTPEVDDE	2560.142	2560.154	5	2+,3+	4,11,13	6,7,8,10,11,13
[109-131]B	(E)-NSAEPEQSLACQCLVRTPEVDDE	2532.111	2532.136	10	2+,3+	7,11,14	1,12,14
[109-134]A	(E)-NSAEPEQSLVCQCLVRTPEVDDEALE	2873.306	2873.316	3	3+,2+	1,2,3,4,6,7,10,12,15	1,2,6,9,10,11
[115-127]A	(E)-QSLVCQCLVRTPE	1474.727	1474.742	10	2+	1,3,4,9,10,11	2,3,4,5,7,8,9,10,11
[115-127]B	(E)-QSLACQCLVRTPE	1446.695	1446.712	12	2+	2,3,4,8,10,11	2,4,5,7,8,9,10,11,12,13
[115-134]B	(E)-QSLACQCLVRTPEVDDEALE	2218.025	2218.028	1	2+	1,5,11	2,10
[128-134]	(E)-VDDEALE	789.339	789.347	10	1+	3,5,6,7	
[135-137]	(E)-KFD	408.201	408.211	24	1+	1,2	1,2,3
[135-141]	(E)-KFDKALK*	848.512	848.519	8	2+,1+	1	1,6,7
[135-157]	(E)-KFDKALKALPMHIRLSFNPTQLE	2696.484	2696.478	-2	3+,4+,2+	1,2,4,5,6,9,11,17(2+), 18(2+),20(2+)	1,2,3,5,6,9,11,20(2+), 21(2+),22(2+)
[135-158]	(E)-KFDKALKALPMHIRLSFNPTQLEE	2825.526	2825.520	-2	3+,4+,2+	1,2,3,4,5,6,9	1,2,3,4,5,6,7,9,11,12,13,15
[135-162]	(E)-KFDKALKALPMHIRLSFNPTQLEEQCHI	3306.737	3306.741	1	3+,4+,2+	1,3,4	2,3,4,5,6,7,8,21(2+),25(2+)
[138-157]	(D)-KALKALPMHIRLSFNPTQLE	2306.293	2306.260	-14	2+,1+	3,5,17(2+),18(2+)	6
[138-158]	(D)-KALKALPMHIRLSFNPTQLEE	2435.336	2435.350	6	3+,2+	3,4,10,15(2+),18(2+),19(2+)	1,6,8
[142-157]	(K)-ALPMHIRLSFNPTQLE*	1865.982	1865.990	4	2+,3+	3,5,11,15(2+)	2,3,5,6,14(2+)
[142-158]	(K)-ALPMHIRLSFNPTQLEE*	1995.025	1995.010	-8	2+,3+	3,5,11(2+),14(2+),15(2+),1 6(2+)	6,7,15(2+)
[146-157]	(M)-HIRLSFNPTQLE*	1453.768	1453.768	0	2+,	2,3,4,5,6,7,9	1,2,3,6,8,10
[146-158]	(M)-HIRLSFNPTQLEE*	1582.810	1582.814	3	2+	2,3,6,7,9	1,6,7,11
[158-162]	(E)-EQCHI	628.264	628.270	10	1+	4,5	2,3,4
[159-162]	(E)-QCHI	499.221	499.229	16	1+	3,4	2

It is important to realize that part of the sequence may be covered by different peptides. Hence, we have defined the peptide sequence coverage as the number of amino acids (#AA) in annotated peptides divided by the total number of amino acids (#AA) present in all peptides (annotated peptides and missing peptides) using equation (5):

(5) $$peptide\ sequence\ coverage = \frac{\#\ AA\ (annotated\ peptides)}{\#\ AA(annotated\ peptides)+\#\ AA\ (missing\ peptides)} \times 100$$

in which missing peptides are defined as those peptides, which should be present given the annotated peptides, but which were not identified in the hydrolysate. (**figure 3**)
A complete amino acid sequence coverage does not necessarily mean a complete peptide sequence coverage, especially in samples that are not completely (i.e. not to 100 % of the maximal DH) hydrolyzed. (**figure 3**)

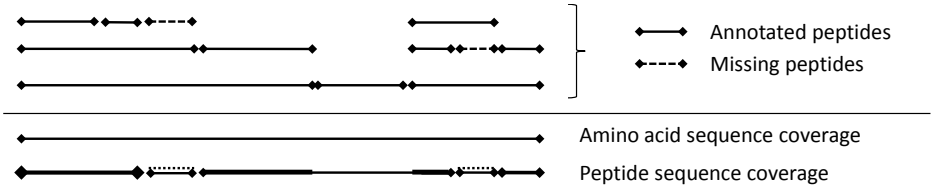

Figure 3. Schematic representation of amino acid sequence coverage and peptide sequence coverage.

Using equation 5, peptide sequence coverages were found to be on average 94 ± 4 % for β-lactoglobulin. This number indicates that only a small number of peptides were missed in the annotation. The values are high (98 %) at low DH and decrease to 90 % with increasing DH. The decrease in peptide sequence coverage with increasing DH is likely due to the formation of short peptides or even single amino acids, which are not (always) annotated or recovered in the chromatogram. By combining the peptides annotated in the samples at each DH, 58 peptides derived from β-lactoglobulin were annotated. (**figure 4A**) In addition to the peptides derived from β-lactoglobulin, 10 peptides were identified as a result of the hydrolysis of α-lactalbumin. The average peptide sequence coverage for peptides derived from α-lactalbumin was found to be 98 ± 1 %. (**figure 4B**)
Out of the 58 peptides derived from β-lactoglobulin identified, 9 peptides are a-specific. (**table 1**) This means that on one or both sides of the peptides, cleavages have not occurred after a glutamic or aspartic acid residue. These peptides are observed in the hydrolysates obtained at DH above DH 4.5 %, indicating that the formation of these peptides results from the cleavage of previously formed (specific) peptides. For instance, the peptides β-lg[56-59] and β-lg[60-62] are formed from the cleavage of the peptide β-lg[56-62]. The same phenomenon is observed for the peptide β-lg[75-89], leading to the peptides β-lg[75-80] and β-lg[81-89], and the peptides β-lg[135-157], β-lg[135-158], β-lg[138-157] and β-lg[138-158], which are further cleaved at the cleavage sites 141 and 145. The cleavages occur after different amino acids (Q, A, K, M) that are

not hydrolyzed in other parts of the sequence (and not in the intact protein). This indicates that the presence of a-specific peptides cannot be attributed directly to the presence of side activity.

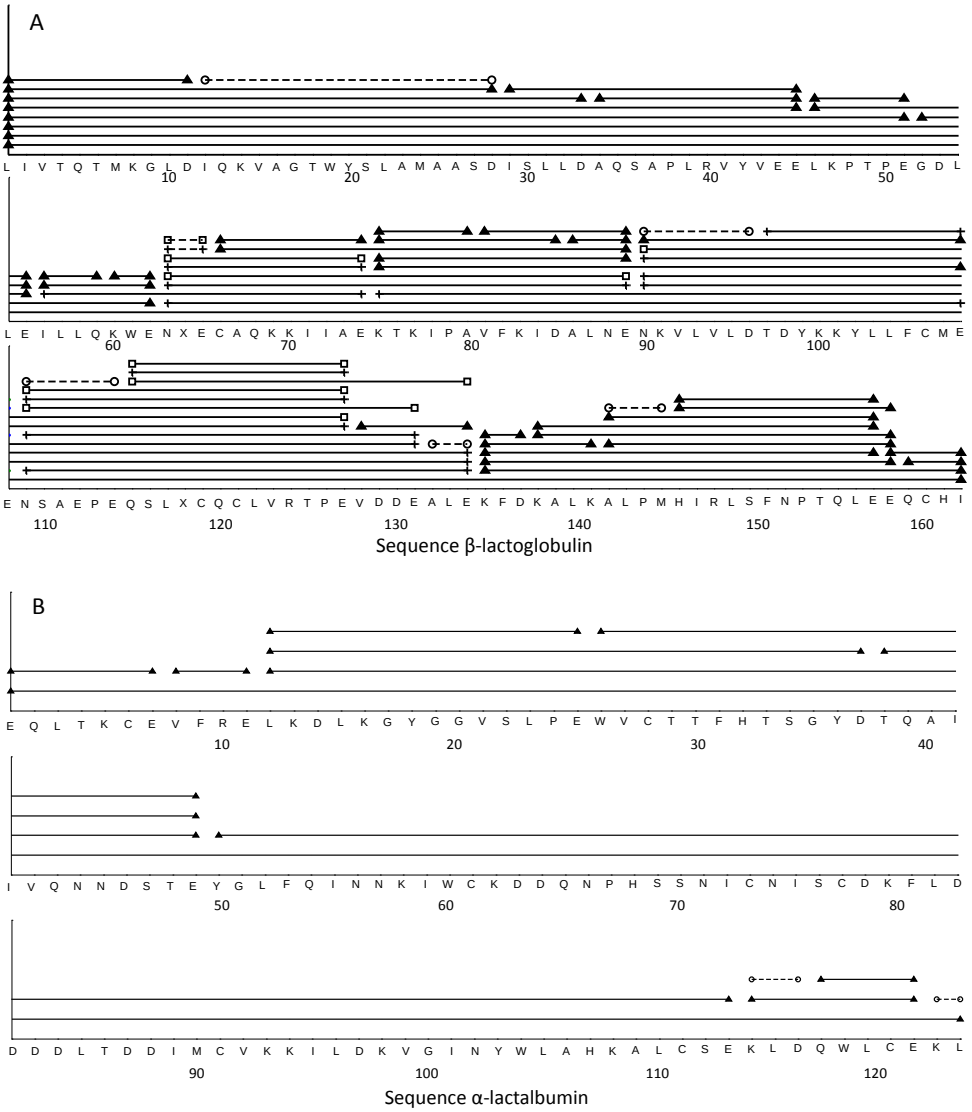

Figure 4. Peptide sequence coverage during hydrolysis of WPI at 1 % (w/v) at different DH for (A) the 58 peptides annotated from the hydrolysis of β-lactoglobulin and (B) the 10 peptides annotated from the hydrolysis of α-lactalbumin. The full lines (▲——▲) represent the annotated peptides and the dotted (○– – –○) lines represent the missing peptides. For β-lactoglobulin, ▲ are used for peptides from both variants, + and □ are used for peptides annotated for variant A and B, respectively. The X in the amino acid sequence refer to amino acids 64 and 118 which differ between β-lactoglobulin variants A and B.

Quantitative sequence coverage

Finding all expected peptides in a given hydrolysate does not guarantee that a complete description of the hydrolysate composition has been accomplished. Actually, every amino acid should be present in a defined amount, which is related to the original amount of protein that is hydrolyzed. The completeness of the hydrolysate description must, therefore, also consider the quantitative recovery of all the peptides that were formed. To quantify peptides, the MS intensities are sometimes used. For three peptides the UV_{214} peak area and the intensity of the MS signal was plotted as function of the concentration. (**figure 5**)

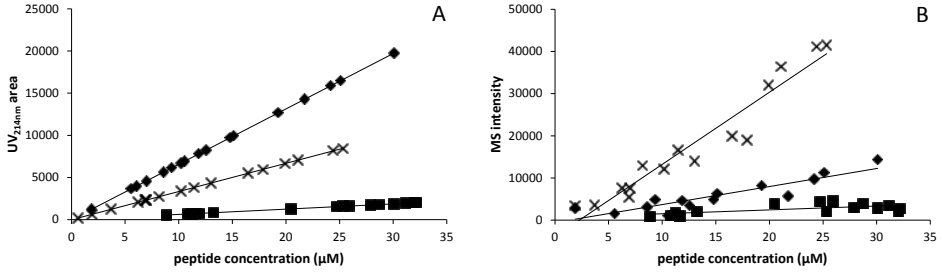

Figure 5. (A) UV_{214} area versus peptide concentration and (B) MS intensity versus peptide concentration for three peptides (♦) β-lg[1-45], (x) β-lg[135-158] and (■) β-lg[159-162].

In both cases, peptide β-lg[159-162] has lower signals than the other peptides. For the other peptides a difference is observed between the different methods. Peptide β-lg[135-158] shows a higher MS intensity than peptide β-lg[1-45] at any concentration, while the UV intensity is lower. This shows that for correct quantification MS signal cannot be used directly. Instead, the UV signal should be used, since the molar UV extinction coefficient (at 214 nm) of any peptide can be calculated [22].

Therefore, after identification of the peptides, the molar concentration of each peptide was determined using the area of the corresponding UV_{214} peak for complete quantification of the hydrolysates. (equation 3) For each sample, UV peaks were included until 90-95 % of the UV_{214} total area was assigned. It was determined that the molar concentration of individual peptides showed a typical standard error of 6 %.

Using the molar concentration of each individual (annotated) peptide $C_{peptide}$, the concentration of each amino acid C_n in that peptide is determined. Next, the concentrations of each amino acid are summed to determine the concentration of each amino acid in the hydrolysate. (**figure 6**) The quality of the recovery, or completeness of the quantification is determined by calculating the molar sequence coverage, using equation (6):

(6) $$Molar\ sequence\ coverage = \left(1 - \frac{\sqrt{\frac{\sum(C_n - C_0)^2}{(\#AA-1)}}}{C_0}\right) \times 100$$

Where C_n and C_0 are the concentrations (µM) of each amino acid n in the protein and the initial concentration of the protein, respectively. #AA is the number of amino acid residues in the sequence. The molar sequence coverage was found to be on average 75 ± 15 % for β-lactoglobulin and 65 ± 15 % for α-lactalbumin, indicating a quite complete annotation and quantification. Still, for certain amino acids a higher and for others a lower concentration is found than expected based on the initial protein concentration. Such small errors could perhaps be solved in future by further optimizing the separation (to avoid co-elution), annotation and quantification. In addition, as mentioned above, in some parts of the sequence the missing signal is probably due to the presence of free amino acids or dipeptides.

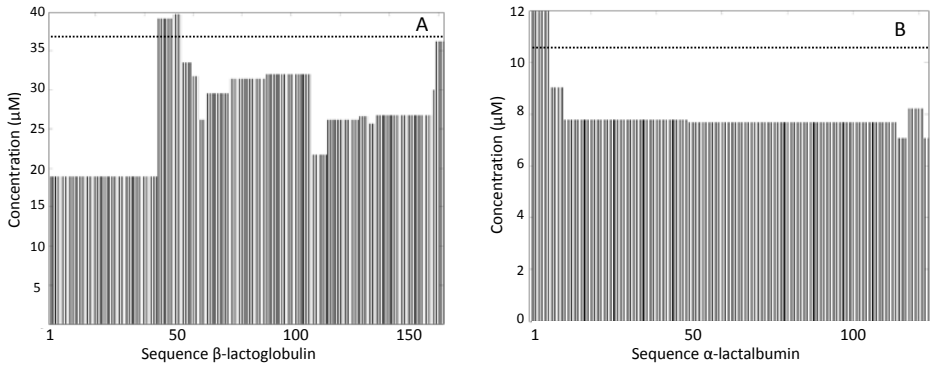

Figure 6. Concentration of all amino acid C_n for (A) β-lactoglobulin for sample 1 % (w/v) WPI at DH 3 % and (B) for α-lactalbumin for sample 1 % (w/v) WPI at DH 4.5 %. The dotted line (·····) shows the initial protein concentration.

Free amino acids and small peptides are usually not detected in RP-UPLC-MS analysis. Based on the specificity of the enzyme BLP, 5 free amino acids are expected from the sequence of β-lactoglobulin (45-E, 65-E, 130-D, 131-E, 157-E). Finally small variation in the quantification resulting from a variation in the total UV area over all samples were noted. This is due to small losses of material during sample preparation or during the elution.

Characterization of the mechanism of hydrolysis

Kinetics of peptide formation

By quantifying individual peptides, their formation and degradation can be followed over time. To illustrate the formation of each peptide, its molar concentration is plotted as a function of the degree of hydrolysis. Examples of peptide concentrations as a function of DH are shown as representation of the three types of kinetics of peptides formation. (**figure 7**) Peptides β-lg[1-45] and β-lg[135-162] can be formed by the breakdown of the intact protein by only one cut in the intact protein. So, they are formed in large amounts

at the beginning of the hydrolysis. Next, their concentrations decrease with time, indicating that they are further hydrolyzed. (**figure 7A**)

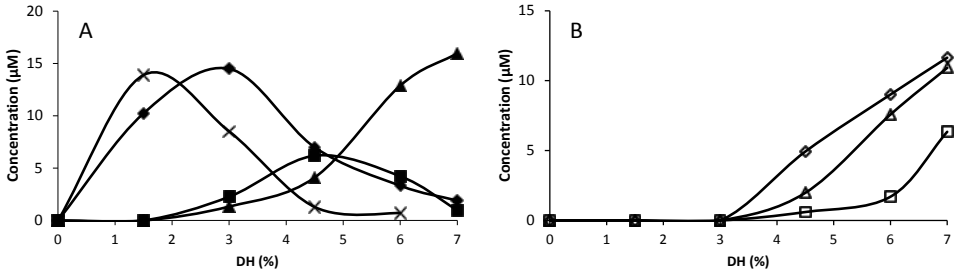

Figure 7. Concentration (µM) of different peptides (A) (♦) β-lg[1-45], (■) β-lg(A)[109-127], (▲) β-lg(A)[115-127], (x) β-lg[135-162] and (B) (◊) α-lac[1-7], (Δ) α-lac[8-11] and (□) α-lac[50-113] as a function of DH for 1% (w/v) WPI hydrolyzed by BLP. All hydrolysates were diluted to 0.1 % (w/v) prior to analysis.

Peptide β-lg(A)[109-127] is first slowly increasing in concentration and then decreasing. It is obtained from at least two cuts on the intact β-lactoglobulin and it is further hydrolyzed producing new peptides. Hence, it is defined as an intermediate peptide. One of the hydrolysis products of β-lg(A)[109-127] is β-lg(A)[115-127]. The concentration of this peptide is increasing as a function of DH. This peptide does not contain any aspartic or glutamic acid residues. So, no further cleavage are expected. This fits with the increasing concentration determined as a function of the DH. It is referred to as final peptide. The peptides from α-lactalbumin are present in very low concentrations at the beginning of the hydrolysis. This was expected since at low DH a large proportion of intact α-lactalbumin remains. (**figures 1B and 7B**) Peptide α-lac[1-7], which can be obtained with only one cut on the intact protein is present in a higher concentration than the others peptides resulting from hydrolysis of α-lactalbumin. Due to the slow hydrolysis of α-lactalbumin, no further degradation of the formed peptides is observed.

Kinetics of cleavage site products formation

The concentration of cleavage products ($C_{i,t}$) that originate from the hydrolysis of the same peptide bond in the parent molecule is calculated using equation (7)

(7) $$C_{i,t} = \sum \left\{ C_{peptide}\left[x-y\right]_t \mid i = x-1 \bigcup i = y \right\}$$

In other words, the concentration of cleavage products formed at each time point t, after hydrolyzing peptide bond no. i equals the sum of all peptides of sequence [x-y], for which i = (x-1) or i = y. This means the peptides that are formed from hydrolysis after the amino acid i or which end by the amino acid i.

Because β-lactoglobulin is the most abundant protein in Bipro and is hydrolyzed faster than α-lactalbumin, only peptides from β-lactoglobulin obtained during hydrolysis of 1 % (w/v) WPI by BLP are considered to determine the enzyme selectivity.

For each cleavage site the apparent cleavage rate ($k_{i,app}$ in s^{-1}) was subsequently calculated by fitting equation (8) to the experimental data. (**figure 8**)

(8) $$C_{i,t} = C_0 - C_0 e^{-k_{i,app} \times t}$$

In which $C_{i,t}$ is the concentration of cleavage products (μM) determined for each time point t and C_0 is the initial concentration of protein (μM).

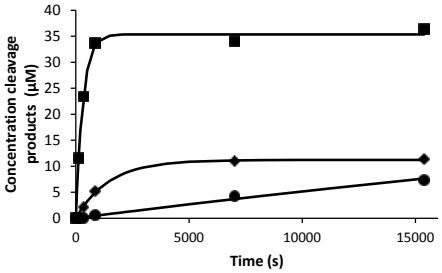

Figure 8. Concentration of β-lactoglobulin cleavage products (●) 33, (■) 74 and (♦) 114 as a function of time obtained during hydrolysis of 1 % WPI by BLP. Markers indicate the experimental data, the line shows the fit using equation (8) (solid line).

The rate of selective hydrolysis k_i ($s^{-1} \cdot mg^{-1}_{enzyme}$) is defined as:

(9) $$k_i = \frac{k_{i,app}}{m_E}$$

in which m_E is the mass, in mg of enzyme added for the hydrolysis.

The rate of selective hydrolysis towards each cleavage site was determined. (equation 10) From these calculations, it became apparent that there were large differences in the rate of hydrolysis towards all cleavages sites, varying from $6 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ to $1.1 \cdot 10^{-6}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ or even 0 for some of the cleavage sites after the same type of amino acids. The cleavage sites were then divided into five groups based on the calculated rate of selective hydrolysis. (**figure 9**) The first group consists of 4 glutamic acid residues for which the enzyme has a rate of selective hydrolysis of $5.5 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. Next, 5 glutamic acid residues belong to the second group, for which the rate of selective hydrolysis was found to be $2.4 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. (**figure 9A**) The rate of selective hydrolysis for the third group (4 glutamic acid residues) is $4.5 \cdot 10^{-4}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. The average rate of selective hydrolysis for the fourth group is $3.0 \cdot 10^{-5}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. The fourth group consists of one glutamic acid residue and 6 aspartic acid residues. It is important to note that two cleavage sites after glutamic acid (44 and 112) and five cleavage sites after aspartic acid residues (53, β-lg(A)-64, 98, 129 and 130) are not hydrolyzed at all, thereby constituting a fifth group. These latter cleavage sites

are all in the neighborhood of other cleavage sites, which might lead to the formation of free amino acids or dipeptides. Free amino acids and short peptides are not always annotated or recovered in the chromatogram. The rate of selective hydrolysis for aspartic acid residues ($3.0 \cdot 10^{-5}$ $s^{-1} \cdot mg^{-1}_{enzyme}$) is on average 1000 times lower than for the glutamic acid residues with the highest rate (rate of selective hydrolysis of $5.5 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$). This confirms previous results determined according to the rate of hydrolysis of synthetic peptides, describing a 1000-fold faster hydrolysis of the cleavage sites after glutamic acid residues than cleavage sites after aspartic acid residues [18].

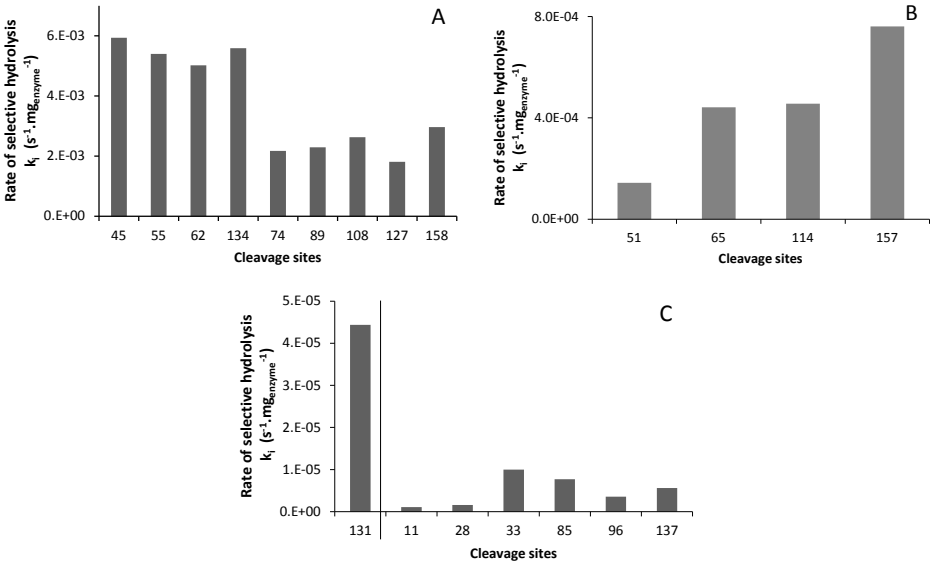

Figure 9. Rate of selective hydrolysis k_i of the enzyme towards glutamic acid and aspartic acid residues of β-lactoglobulin during hydrolysis of 1 % (w/v) WPI by BLP with (A) Glu with high and intermediate rate of selective hydrolysis, (B) Glu with low rate of selective hydrolysis and (C) Glu with very low rate of selective hydrolysis and Asp.

Determination of the selectivity

The $selectivity_{\beta\text{-}lg}$ (%) of the enzyme is calculated by dividing the rate of selective hydrolysis k_i of each individual cleavage site by the sum of the rate of selective hydrolysis of all cleavage sites as in equation (10):

(10) $$Selectivity_{\beta-lg}\ (\%) = \frac{k_i}{\sum k_j} \times 100$$

The selectivity was determined for all cleavage sites, using equation 10. The selectivity varies from 0.003 % to 15 %. (**table 2**) The different cleavage sites are divided into the same group as for the rate of selective hydrolysis, from very high to low selectivity. The enzyme has a very high selectivity (± 15 %) towards 4 Glu residues in the first group.

The selectivity towards the second group (5 Glu residues) is on average ± 6.6 %. The selectivity for the third group with 4 Glu residues is on average ± 1.3 %. Finally the enzyme has a selectivity of ± 0.015 for the residues (1 Glu and 6 Asp) in the fourth group. 7 cleavages sites (2 Glu and 5 Asp) are not hydrolyzed at all, forming the fifth group. (**table 2**)

Table 2. Selectivity$_{\beta\text{-lg}}$ (%) of the enzyme towards all cleavage sites for 1 % (w/v) WPI.

	Cleavage site	Selectivity$_{\beta\text{-lg}}$
Glu with high selectivity	45	17
	55	15
	62	14
	134	16
Glu with intermediate selectivity	74	6
	89	6
	108	7
	127	5
	158	8
Glu with low selectivity	51	0.40
	65	1.2
	114	1.3
	157	2.1
Glu with very low selectivity - Asp residues	131	0.035
	11	0.0031
	28	0.0044
	33	0.028
	85	0.022
	96	0.0099
	137	0.016
Non-hydrolyzed Glu and Asp residues	44	n.d.
	112	n.d.
	53	n.d.
	A-64	n.d.
	98	n.d.
	129	n.d.
	130	n.d.

n.d.: Peptides were analyzed until a DH of 7 %, for certain cleavage sites no corresponding peptides were found, indicating that the selectivity is < 0.003 %.
Note: The sum of selectivities of all cleavage sites is 100 %.

The division into groups clearly shows that while the enzyme is specific for glutamic acid residues, it has different selectivities (i.e. preference) towards the different glutamic acid residues. To understand why the enzyme has a high or intermediate selectivity for 9 cleavage sites after glutamic acid residues in β-lactoglobulin and low or very low selectivity for the other 7 glutamic acid residues, several hypotheses can be considered based on the tertiary, secondary and primary structure of the intact protein.
Based on the protein structure, the accessibility of all glutamic acid residues is expected to be similar. Firstly, they are found on the outside of the protein, since they are negatively charged. Secondly, β-lactoglobulin is usually present as a dimer [24,25] in solution, but none of the 16 glutamic acid residues are involved in the dimer interface of β-lactoglobulin or close to this interface [26]. Consequently, the dimerization of the

molecule does not play a role on the physical accessibility of the glutamic acid residues and does not explain the selectivity. Moreover, the glutamic acid residues are found both on α-helices and β-sheets.

The enzyme BLP does not favor any type of secondary structure, while it has been suggested in a different study that α-helices in the β-lactoglobulin structure hinder the physical approach of trypsin [11]. One indication for a difference is obtained by comparing the amino acids that are positioned after the glutamic acid residues on position P1', thus the closest to the peptide bond to be hydrolyzed. The positively charged residues (two lysine residues) are never found after cleavage sites with low selectivity. In contrast the negatively charged ones (two glutamic acid residues) are always found after cleavage sites with low selectivity. The cleavage site 44, next to Glu-45, is indeed not cleaved at all. This might indicate some influence of charged residues at the C-terminus of the cleavage sites even if other factors are certainly affecting the cleavage rates. Also, it should be noted that cleavage site 112 is also not cleaved. The residue on position P1' is a proline and it has been previously shown that this amino acid can hinder the action of BLP [5].

Not all putative cleavage sites next to the same type of amino acid in the protein are hydrolyzed at the same rate as described by the large range of selectivity calculated, from 17 % to 0.003 %. This shows the importance of defining the enzyme in terms of selectivity rather than specificity, to describe quantitatively which cleavage sites are preferred.

Conclusions

Using a quantitative description of the peptides present in an hydrolysate, the selectivity of a protease for a certain cleavage site in the substrate can be quantitatively determined. It clearly showed large differences in terms of enzyme selectivity towards all cleavage sites after the same type of amino acid residue. Selectivity can be used as descriptor of enzymatic hydrolysis to increase the understanding of the influence of hydrolysis conditions.

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Chapter 5

Influence of substrate concentration on the enzyme selectivity

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Abstract

An increase of the substrate concentration during enzymatic protein hydrolysis has often been found to result in a decrease in the rate of hydrolysis even at constant enzyme to substrate ratio. It is not known if only the rate is affected, or if the mechanism of hydrolysis is affected as well. To describe changes in the mechanism of hydrolysis, the selectivity of the enzyme towards the different cleavage sites is determined. The selectivity is defined as the relative rate of hydrolysis of each cleavage site in the protein divided by the hydrolysis rate of all cleavage sites. The selectivity is determined from the identification and quantification - in terms of molar concentration - of the peptides present in the hydrolysates at different degrees of hydrolysis. To test the effect of concentration on the enzyme selectivity, solutions of 0.1-10 % (w/v) whey protein isolate (WPI) were hydrolyzed by *Bacillus licheniformis* protease at constant enzyme to substrate ratio. The Glu and Asp cleavage sites of β-lactoglobulin were divided into 5 groups based on the selectivity of the enzyme from very high selectivity (> 10 %) to very low selectivity (< 0.1 %) towards these cleavage sites for all initial substrate concentrations. The enzyme selectivity towards the cleavage sites after Glu 62 and 134, is two times higher at 10 % (w/v) WPI than at the lower protein concentrations. Some influence of substrate concentration towards Glu cleavage sites with lower selectivity is also noted but these have less influence on the mechanism of hydrolysis. Furthermore, the enzyme has a higher selectivity towards cleavage sites next to Asp residues at high protein concentrations (5-10 % (w/v)) WPI than at low protein concentrations (≤ 1 % (w/v) WPI). This shows that both the rate of hydrolysis and the enzyme selectivity are influenced by the substrate concentration.

Submitted

Introduction

Enzymatic protein hydrolysis is usually performed at protein concentrations below 10 % (w/v). By increasing the concentration, and consequently reducing the amount of water, the energy consumption of the process might be reduced. However, increasing the substrate concentration generally results in lower hydrolysis rates and lower values of the final degree of hydrolysis [1]. Typically, this is shown in studies with a constant enzyme concentration independent of the substrate concentration. For instance, the hydrolysis of rapeseed protein isolate showed a decrease in the final degree of hydrolysis (DH) from 19 to 6 % with an increase in substrate concentration from 5 to 130 $g \cdot L^{-1}$ [2]. In addition, hydrolysis of micellar casein at elevated concentrations from 40 to 85 $g \cdot L^{-1}$ showed a decrease from 15 to 10 % DH after two hours of hydrolysis [3]. Even if the enzyme to substrate ratio is maintained constant, higher substrate concentrations lead to lower DH values [4]. Hence, the picture emerges that this negative influence of increasing initial substrate concentration on protein hydrolysis kinetics is independent of the substrate or the enzyme. Surprisingly little attention has been given to this. Consequently, the reason for this phenomenon is not well understood. It has been suggested that a higher solvent viscosity will result in a lower reaction rate [5]. In recent work, however, the influence of viscosity was shown not to be the reason for the observed decrease in DH [4]. Other explanations could be sought in changes in the structure or structural stability of the substrates and enzymes used, or in the solvent quality. Such changes could lead to differences in the accessibility of the substrate. However, it was shown for β-lactoglobulin that an increase in concentration only had a relatively minor effect on the structural stability of the intact protein [6].

Another explanation is that the mechanism of hydrolysis is affected by the substrate concentration resulting in differences in hydrolysis rate. Several studies have indicated differences in the mechanism of hydrolysis at different substrate concentrations. For instance, the peptide profiles of whey protein concentrate (WPC) hydrolyzed by Corolase PP at substrate concentrations of 10 and 15 % (w/v) at constant enyzme/substrate ratio (4/100 w/w) after 5 hours of hydrolysis showed more free amino acids obtained at 15 % WPC than at 10 % and a larger proportion of intermediate and high molecular weight peptides at 10 % WPC than at 15 % [7]. Another study showed that a particular peptide (responsible for bitterness) was formed more at 50 $g \cdot L^{-1}$ than at 300 $g \cdot L^{-1}$ initial whey protein concentration at DH 15 % [8]. While these observations may be taken as an indication for changes in the hydrolysis mechanism, for a better understanding the peptide profile of the hydrolysates should be analyzed in much greater detail. The mechanism of hydrolysis can be described by the selectivity of the enzyme. The selectivity has recently been defined as the relative rate at which the enzyme cleaves each individual cleavage site compared to the total rate of hydrolysis of all cleavage sites in the protein (chapter 4).

Furthermore, it has already been shown that there are large differences in terms of selectivity towards cleavage sites after the same type of amino acid for the hydrolysis by *Bacillus licheniformis* protease (BLP) of a 1 % (w/v) WPI solution (chapter 4). In the

present study, the same specific enzyme (BLP) is used for the hydrolysis of 0.1-10 % (w/v) whey protein isolate (WPI). The molar concentration of most of the peptides (i.e.> 90 % of the total UV_{214} signal) in the hydrolysates was determined, at different points during the hydrolysis. From the kinetics of formation of the peptides, the influence of substrate concentration on the selectivity of the enzyme was determined.

Materials and Methods

Materials. Bipro, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). It contained (by weight) 74.0 % β-lactoglobulin, 12.5 % α-lactalbumin, 5.5 % bovine serum albumin and 5.5 % immunoglobulin, according to specifications of the supplier. The protein content (Nx6.32) [4] of the powder was 93.4 % (w/w) as determined by Dumas. BLP (*Bacillus licheniformis* protease), specific for Glu-X bonds and for Asp-X bonds [9], was obtained from Novozymes (NS-37005) (Bagsvaerd, Denmark). BLP (4.5 % protein, Nx6.25, 0.3 AU/mg/min as determined by the azocasein assay [10]) was partly insoluble and was fractionated as described before (chapter 3). The freeze dried material was found to contain 60 % protein (Nx6.25) from which 78 % is the enzyme BLP (23.6 kDa) and 14 % is the pro-peptide (6.9 kDa) based on UV_{214}, as determined by RP-UPLC-MS (chapter 3). An activity of 3.9 AU/mg/min was determined by the azocasein assay. All other chemicals were of analytical grade and purchased from Sigma or Merck.

Hydrolysis

Protein solutions were prepared by dispersing WPI powder at a concentration of 45 % (w/v) in Millipore water, followed by stirring overnight at 4 °C. Insoluble parts were removed by centrifugation (30 min, 4000 g, 20 °C) and the supernatant obtained (30 % (w/v)) was diluted to the required final concentrations 0.1-10 % (w/v) as determined by UV_{280} [4]. Hydrolyses were performed using a pH-stat. Solutions of WPI at 0.1, 0.25, 0.5, 1, 5 and 10 % (w/v) were hydrolyzed at 40 °C and pH 8 using NaOH at concentrations of 0.02, 0.05, 0.1, 0.2, 1 and 2 M, respectively, to keep the pH constant. Protein solutions (10 mL) were equilibrated at least 15 minutes at 40 °C and adjusted to pH 8.0 before addition of BLP dissolved at 5 % (w/v) in Millipore water (0.30 µL of enzyme/ mg of protein). The degree of hydrolysis (DH) was calculated based on the added volume of NaOH using 1/α = 1.20 at 40 °C and pH 8.0 and h_{tot}(WPI) = 8.5 as described before [4], (chapter 3).

The overall hydrolysis rate k_{hydr} was calculated using equation (1)

(1) $$DH = \frac{1}{a}\ln(1 + k_{hydr}.t)$$

In which k_{hydr} and *a* are fitting parameters for the overall hydrolysis rate (s^{-1}) and substrate inactivation, respectively, and t the time in seconds [4]. The overall relative hydrolysis rate was determined using the highest value of overall hydrolysis rate as 100 %. The overall hydrolysis rates of BLP were compared with previous results obtained with Alcalase [4].

Samples were taken during the hydrolysis of 0.1, 0.5, 1, 5 and 10 % (w/v) protein solutions at different degrees of hydrolysis (1.5, 3, 4.5, 6 and 7 %). These samples were then centrifuged (5 min, 19000 g, 15 °C). Supernatant and pellet were separated, and the supernatants were inactivated by adjusting the pH to 2 with 5 M HCl. The pH was set back to 8.0 after at least 10 minutes of inactivation before storage of the samples at -20 °C.
Samples obtained during hydrolysis of 0.1 % (w/v) and 0.5 % (w/v) WPI were freeze dried after the pH was re-adjusted to pH 8.0, and re-dissolved to a concentration of 1 % (w/v) to have the same concentration for all sample for analysis on RP-UPLC-ESI-MS.

Solubility

The protein contents in the supernatants of the samples taken at different DH values were determined using DUMAS (Nx6.32) [4] using a Flash EA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The proportion of soluble protein was calculated by dividing the concentration of protein of the supernatants by the concentration of protein in the solution before centrifugation at DH = 0.

Reverse Phase Ultra High Performance liquid chromatography (RP-UHPLC)

Samples obtained during hydrolysis were analyzed on an Acquity UPLC System (Waters, Milford, MA, USA) using an Acquity UPLC BEH 300 C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 x 50 mm, 1.7 µm particle size). Eluent A was 1 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) in Millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. To reduce disulfide bridges and to facilitate peptide annotation, samples were first incubated for two hours with 100 mM dithiothreitol (DTT) in 50 mM Tris·HCl, pH 8.0 at a concentration of 0.5 % (w/v). After incubation, samples were further diluted in eluent A to a final concentration of 0.1 % (w/v) and centrifuged (10 min, 19000 g, 20 °C). Samples (4 µL) were injected into the column thermostated at 40 °C (chapter 4).
The amount of remaining intact proteins (β-lactoglobulin, A and B, and α-lactalbumin) was determined using the following elution profile: 0-2 min isocratic on 30 % B; 2-12 min linear gradient from 30 % B to 50 % B; 12-15 min linear gradient from 50 to 100 % B; 15-20 min isocratic on 100 % B; 20-21 min from 100 % B to 30 % B and 21-30 min isocratic on 30 % B. The proportion of remaining intact α-lactalbumin and β-lactoglobulin was determined by dividing the area of the UV_{214} peak obtained for each protein at different DH values by the peak area obtained for each intact protein before hydrolysis. The proportion of β-lactoglobulin takes into account the proportion of both A and B variants.
For separation of the peptides a different elution profile was used: 0-2 min isocratic on 3 % B; 2-10 min linear gradient from 3 % to 22 % B; 10-16 min linear gradient 22 - 30 % B; 16-19 min linear gradient 30-100 % B; 19-24 min isocratic on 100 % B; 24-26 min linear gradient 100-3 % B and 26-30 min isocratic on 3 % B. The flow rate was 350

µL·min^{-1}. Detection was performed using a PDA, which scanned the absorbance from 200-400 nm at a 1.2 nm resolution, with 20 points per second.

Electron spray ionization time of flight mass spectrometry (ESI-Q-TOF-MS)

The mass spectra of the hydrolysates were determined with an online Synapt high definition mass spectrometer (Waters), coupled to the RP-UPLC system, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). The system was calibrated using Glu-1-Fib. The capillary voltage was set to 3 kV with the source operation in positive ion mode and the source temperature at 120 °C. The sample cone was operated at 35 V. Nitrogen was used as desolvation gas (250 °C, 800 L/h) and cone gas (200 L/h). The trap gas was set at 1.5 mL/min. MS and MS/MS (MSe method) were performed between m/z 100-2000 with a scan time of 0.3 seconds. The trap collision energy was set at 6 V in single MS mode and ramped from 20 to 30 V in MSe mode. The transfer collision energy was set at 4 V in MS and switched between 4 and 10 V in the MSe mode. UV and MS data were acquired using MassLynx v4.1 (Waters).

Peptide identification and quantification

For peptide identification, internal lock mass was applied on every chromatogram for MS and MS/MS data with two previously identified masses chosen among the most abundant masses, of which the sequences were identified manually. The identification of MS and MSe ion peaks was done by Biopharmalynx software (v1.3) with the parameters described before (chapter 4). The annotation was first done using a method to identify peptides cleaved after Glu and Asp residues. Next, a generic method was used in which no enzyme specificity was selected. For UV peaks where the automatic annotation did not yield a peptide, a manual analysis of the MS and MS/MS data was used to assign the peptide. For the total peptide quantification, peaks in the UV_{214} chromatogram were included until 90-95 % of the total UV_{214} area was included. Peptides from β-lactoglobulin and α-lactalbumin as well as the intact protein were quantified using the UV_{214}. In case of co-eluting peptides, the UV peak area was divided over the co-eluting peptides based on the MS intensity (chapter 4). For the further determination of the selectivity only peptides from β-lactoglobulin were considered.

The quantification of the peptides was based on the UV signal at 214 nm, using equation (2):

(2) $$C_{peptide} = 1 * 10^6 \left(\frac{A_{214}}{\varepsilon_{214} l V_{inj} k_{cell}}\right) Q$$

In which $C_{peptide}$ (µM) is the concentration of peptide, A_{214} (AU.min) is the UV peak area at 214 nm, V_{inj} (µL) is the volume of sample injected, Q the flow rate in µL·min^{-1}, l is the path length of the UV cell which is 1 cm according to the manufacturer. The value for the cell constant k_{cell} was previously determined to be 0.66 using pure peptides, but

depends on the geometry of the UV detector used [11]. The molar extinction coefficient at 214 nm of each peptide (ε_{214}) was calculated as described before [12].

Quality of the annotation and of the quantification

For each hydrolysate, the quality of the peptide identification and of the quantification was described by three different parameters. First, the amino sequence coverage was determined by calculating the number of unique annotated amino acid divided by the total number of amino acids present in the parental protein sequence [13]. The amino acid sequence coverage was 100 % for most samples. Only for samples DH 6 and 7 % at 5 and 10 % (w/v) WPI an amino acid sequence coverage of 95 % was obtained. To take into account the fact that each part of the sequence is covered by several peptides, the peptide sequence coverage was determined as a second quality parameter on the peptide annotation (chapter 4). It is defined as the number of amino acids (# AA) in annotated peptides divided by the total number of amino acids (#AA) present in all peptides (annotated peptides and missing peptides) as in equation (3):

(3) $$peptide\ sequence\ coverage = \frac{\#\ AA\ (annotated\ peptides)}{\#\ AA(annotated\ peptides)+\#\ AA\ (missing\ peptides)} \times 100$$

The peptide sequence coverage was found to be on average 95 ± 3 % for all hydrolysates.

To determine the completeness of the quantification, the molar sequence coverage is calculated. For that, the concentration of each amino acid C_n (µM) was calculated using the molar concentration of each individual (annotated) peptide $C_{peptide}$ (µM). With this, each individual amino acid of the primary sequence of β-lactoglobulin has been quantified in the hydrolysate. The molar sequence coverage is calculated using equation (4):

(4) $$Molar\ sequence\ coverage = \left(1 - \frac{\sqrt{\frac{\sum(C_n - C_0)^2}{(\#AA-1)}}}{C_0}\right) \times 100$$

Where C_n and C_0 are the concentrations (µM) of each amino acid n in the protein and the initial concentration of the protein, respectively. #AA is the number of amino acid residues in the sequence.

Determination of the enzyme selectivity

To describe the selectivity of the enzyme, the rate at which individual peptide bonds are hydrolyzed (e.g. between Glu-45 and Leu-46), was determined. To do this, first the concentrations of all peptides originating from hydrolysis of the same peptide bond ($C_{i,t}$) in the parent molecule are calculated using equation (5)

(5) $$C_{i,t} = \sum \left\{ C_{peptide}\left[x-y\right]_t \mid i = x-1 \bigcup i = y \right\}$$

In other words, the concentration of cleavage products formed at each time point t, after hydrolyzing peptide bond no. i equals the sum of all peptides of sequence [x-y], for which i = (x-1) or i = y. This means the peptides that are formed from hydrolysis after the amino acid i or which end by the amino acid i.

For each cleavage site the apparent cleavage rate ($k_{i,app}$ in s^{-1}) was then calculated by fitting equation (6) to the experimental data.

(6) $C_{i,t} = C_0 - C_0 e^{-k_{i,app} \times t}$

In which $C_{i,t}$ (µM) is the concentration of cleavage products determined for each time point t and C_0 is the initial concentration of protein (µM).

The rate of selective hydrolysis k_i ($s^{-1} \cdot mg^{-1}_{enzyme}$) is defined as:

(7) $k_i = \frac{k_{i,app}}{m_E}$

in which m_E is the mass of enzyme added for the hydrolysis (in mg). Subsequently, the $selectivity_{\beta\text{-lg}}$ (%) is calculated by dividing the rate of selective hydrolysis k_i of each individual cleavage site by the sum of the rate of selective hydrolysis of all cleavage sites at each protein concentration as in equation (8):

(8) $Selectivity_{\beta-lg}\ (\%) = \frac{k_i}{\sum k_j} \times 100$

Reproducibility of annotation and quantification

To test the reproducibility of the quantification method, one sample (1 % WPI, DH 4.5 %) was injected 3 times. The concentration determined for individual peptides showed a typical standard error of 6 %. In addition, the reproducibility of the complete analysis was tested. For this, two separate hydrolysis experiments were performed at the same protein concentration (0.5 % (w/v) WPI). During each hydrolysis, samples were taken at DH 1.5, 3, and 6 %. Peptides were annotated and quantified for all hydrolysates. The error of peptide sequence coverage and molar sequence coverage was calculated for the hydrolysate at each DH. The values of errors were averaged to determine the analysis error. The quality of the annotation as described by the peptide sequence coverage showed a standard error of 2.5 %. The molar sequence coverage showed a standard error of 18 %. The determined selectivity showed a typical standard error of 15 %.

Results and Discussion

Protein hydrolysis at different substrate concentrations

The degree of hydrolysis (DH) of WPI hydrolyzed by BLP reached after 10,000 s decreases with increasing initial protein concentration from DH=14 % for 0.1 % (w/v) WPI, to DH = 6 % for 10 % (w/v) WPI. (**figure 1A**) The effect of concentration observed here for WPI hydrolyzed by BLP was compared to previous results obtained for hydrolysis of WPI by Alcalase, an enzyme mixture that mostly contains the a-specific enzyme Subtilisin [4]. In both cases the overall hydrolysis rate k_{hydr} decreases with increasing substrate concentration. (**figure 1B)**

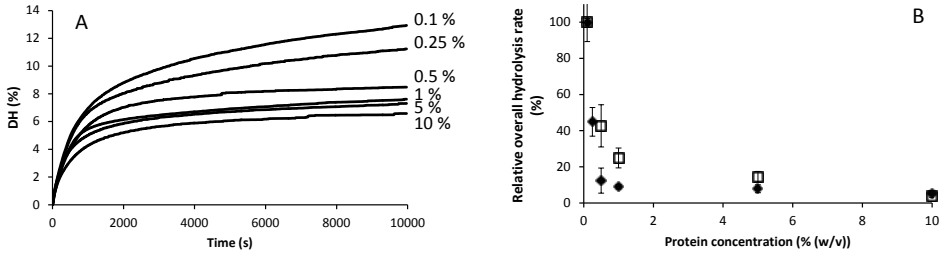


Figure 1. (A) DH curves of 0.1-10 % (w/v) WPI hydrolyzed by BLP at E/S constant and (B) Relative overall hydrolysis rate k_{hydr} for hydrolysis of WPI by BLP (♦) and by Alcalase (□) as a function of initial WPI concentration at constant E/S.

The solubility of all the hydrolysates was determined to be 96 % ± 5 %. This shows that the effect of the initial substrate concentration on the overall hydrolysis rate is not due to the formation of insoluble aggregates. It also indicates that the peptides identified represent the complete sample. Based on this, the pellet was not analyzed for peptides. The hydrolysates obtained at different concentrations can thus be compared for detailed analysis of the hydrolysates and peptides formed as a function of initial protein concentration.

The hydrolysis was also characterized by the decrease of intact proteins as a function of the DH. Changes in this curve indicate changes in the preference for the hydrolysis of intact proteins versus the hydrolysis of intermediate peptides formed. These curves are found to be similar for all initial protein concentrations (0.1-10 % (w/v) WPI), with no remaining intact β-lactoglobulin above DH = 4.5 %. (**figure 2**) In other words, the accessibility towards the intact protein relative to the accessibility towards the peptides is not affected by the initial substrate concentration.

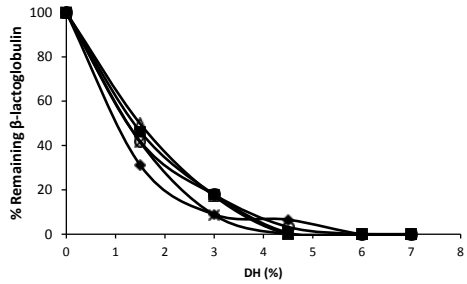


Figure 2. Remaining intact β-lactoglobulin as a function of DH for different initial substrate concentrations (♦) 0.1 %, (■) 0.5 %, (Δ) 1 %, (x) 5 % and (○) 10 % (w/v) WPI.

Both the solubility of the hydrolysates and the difference in accessibility of the intact protein relative to that of peptides are independent of the initial protein concentration while the overall hydrolysis rate is affected by the initial protein concentration. To determine if the mechanism of hydrolysis depends on the initial substrate concentration, the peptides formed during the hydrolysis were identified.

Identification and quantification of the peptides

In total for all samples at different DH and different initial substrate concentrations, 77 peptides were identified and quantified. (**Annexes 1 and 3**) The quality of the quantification is determined by calculating the molar sequence coverage. The molar sequence coverage provides an indication of the concentration of each amino acid of the parental protein sequence found in the peptides identified compared to the initial protein concentration. (equation 4) The molar sequence coverage is 70 ± 10 %, indicating a quite complete annotation and quantification. Sill, for some amino acids, a concentration higher than expected is obtained and for others a concentration lower than expected is determined. (**figure 3**)

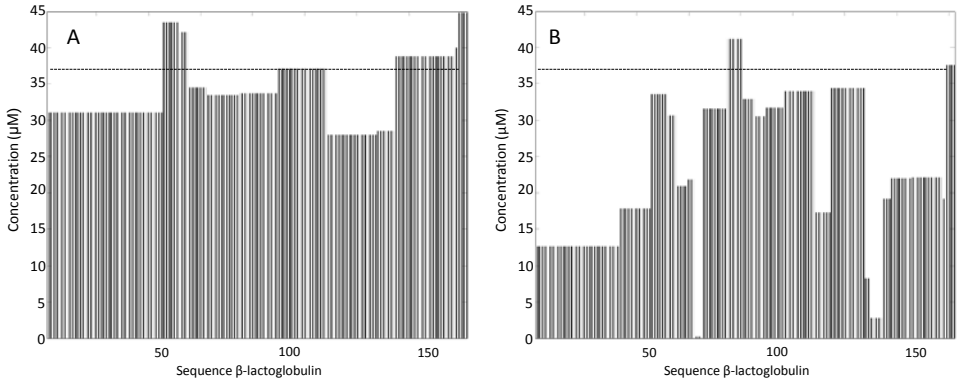


Figure 3. Concentration of all amino acids (C_n) for (A) 1 % (w/v) WPI at DH 1.5 % and (B) 5 % (w/v) WPI at DH 6 %. The dotted line (·····) indicates the initial protein concentration.

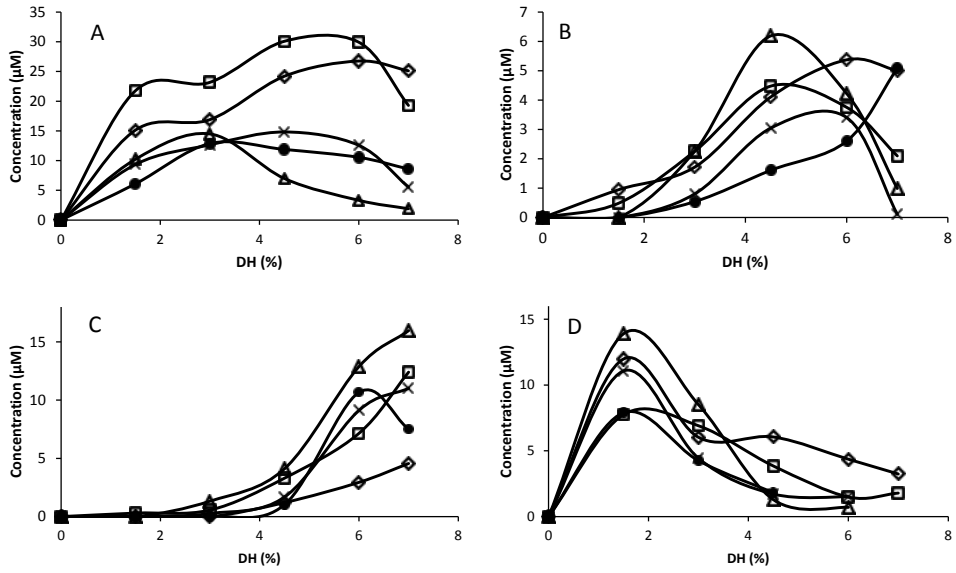
At increasing DH, in some part of the sequence not all amino acid are present to the expected concentration or are not annotated at all. (**figure 3B**) This is most likely due to the formation of free amino acids or short peptides, which are not annotated in RP-UPLC. In total, 5 free amino acids can be obtained during hydrolysis of β-lactoglobulin by BLP (45-E, 65-E, 130-D, 131-E, 157-E). This is probably also the reason why a lower amino acid sequence coverage was obtained for samples at DH 6 and 7 % at 5 and 10 % (w/v) WPI.

Over all hydrolysates, 13 out of the 77 peptides annotated are so-called a-specific peptides. These peptides are the result of cleavage of previously formed (specific) peptides. For instance the formation of β-lg[56-59] and β-lg[60-62] results from the cleavage of the specific peptide β-lg[56-62]. (**table 1**) The formation of a-specific peptides is actually increased with increasing substrate concentration and DH, while the overall hydrolysis rate decreases. Since at higher protein concentrations longer incubation times are needed to reach a certain DH, it is concluded that the formation of these a-specific peptides is correlated with the incubation time rather than with the enzyme activity (chapter 7).

Table 1. A-specific peptides annotated during enzymatic protein hydrolysis of 0.1-10 % (w/v) WPI by BLP and the parental peptides.

Parental peptides	Sequence	A-specific peptides annotated
[56-62]	ILLQ – KWE	[56-59] - [60-62]
[75-89]	KTKIPA – VFKIDALNE	[75-80] - [81-89]
[135-157]	KFDKALK – ALPMHIRLSFNPTQLE	[135-141] - [142-157]
[135-158]	KFDKALK – ALPMHIRLSFNPTQLEE	[135-141] - [142-158]
[138-157]	KALKALPM – HIRLS – FNPTQLE	[138-145] - [146-157] and [146-150] - [151-157]
[138-158]	KALKALPM – HIRLS – FNPTQLEE	[138-145] - [146-158] and [146-150] - [151-158]

The formation and further breakdown of all peptides was monitored as a function of the DH for the different initial protein concentrations. Different types of peptide release kinetics are observed as function of the initial protein concentration. (**figure 4**)



(A) β-lg[1-45] LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE
(B) β-lg(A)[90-127] (E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPE
(C) β-lg(A)[115-127] (E)-QSLVCQCLVRTPE
(D) β-lg[135-162] (E)-KFDKALKALPMHIRLSFNPTQLEEQCHI

Figure 4. Concentration (µM) of different peptides as a function of DH for different initial protein concentration, (◊) 0.1 %, (□) 0.5 % (Δ) 1 % (x) 5 % and (●) 10 % (w/v) WPI. All hydrolysates were diluted to 0.1 % (w/v) prior to analysis.

For some peptides no significant effect of substrate concentration on their formation and subsequent hydrolysis is observed, e.g. peptide β-lg[135-162]. (**figure 4D)** Others, such as peptide β-lg[1-45], show two types of kinetics for low (0.1-0.5 % (w/v) WPI) and for high initial substrate concentrations (1-10 % (w/v) WPI). (**figure 4A**) For peptides, such as β-lg[1-45], a higher peptide concentration (25-30 µM) is reached at low substrate concentrations (< 1 % WPI) than at high substrate concentrations (12 µM). This indicates a faster rate of formation than degradation at low substrate

concentrations and a comparable rate of formation and degradation at high substrate concentrations. In other words, the peptide is less accumulated at high initial substrate concentrations. (**figure 4A**) For the final peptide β-lg(A)[115-127], no large effect of initial substrate concentration on its formation is observed. (**figure 4C**)

Influence of the substrate concentration on the selectivity

Kinetics of hydrolysis

Based on the peptide identification and quantification, the concentration of cleavage products $C_{i,t}$ is determined. (equation 5) The concentration of cleavage products, e.g. 45 and 157 as a function of DH can be compared for the different initial substrate concentrations. (**figure 5**)

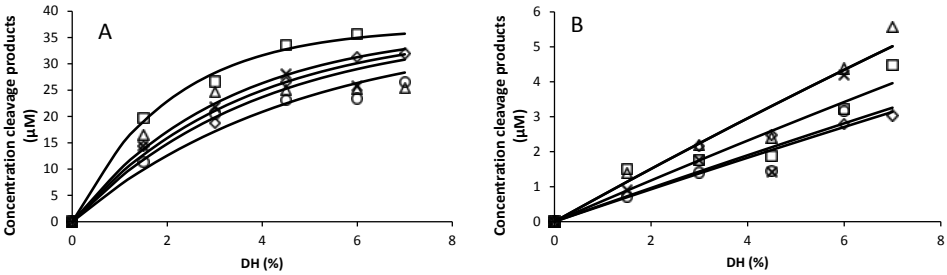


Figure 5. Concentration of cleavage products as a function of DH for (A) cleavage site 45 and (B) cleavage site 157 for the different initial protein concentrations (◊) 0.1 %, (□) 0.5 %, (Δ) 1 %, (x) 5 % and (○) 10 % (w/v) WPI. Markers indicate experimental data and the solid lines show the fits from equation 6.

The concentration of cleavage products is comparable for all initial substrate concentrations. The concentration is higher for cleavage site 45 than for cleavage site 157. The apparent cleavage rate $k_{i,app}$ and rate of selective hydrolysis k_i of each cleavage site are determined based on the concentration of cleavage products as a function of time (equations 6 and 7). The apparent cleavage rate is determined by fitting a first order kinetic equation. For most of the cleavage sites the fit is good. However, the fit is not as good for cleavage sites hydrolyzed at a later stage of the hydrolysis. Still, using a different fit would only introduce more fitting parameters and inaccuracies. Hence, the first order kinetics fit was the most efficient. Large differences in the rate of selective hydrolysis are observed for all cleavage sites over all protein concentrations with rates varying from $2.5 \cdot 10^{-2}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ to $1.0 \cdot 10^{-7}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ and even 0 for some of the cleavage sites. (**figure 6**)

Based on the rate of selective hydrolysis of 1 % (w/v) WPI, the cleavage sites are divided in five groups. The first group consists of 4 Glu residues with a rate of selective hydrolysis $6.5 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. The second group consists of 5 Glu residues with a rate of selective hydrolysis of $2.4 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. (**figure 6A**)

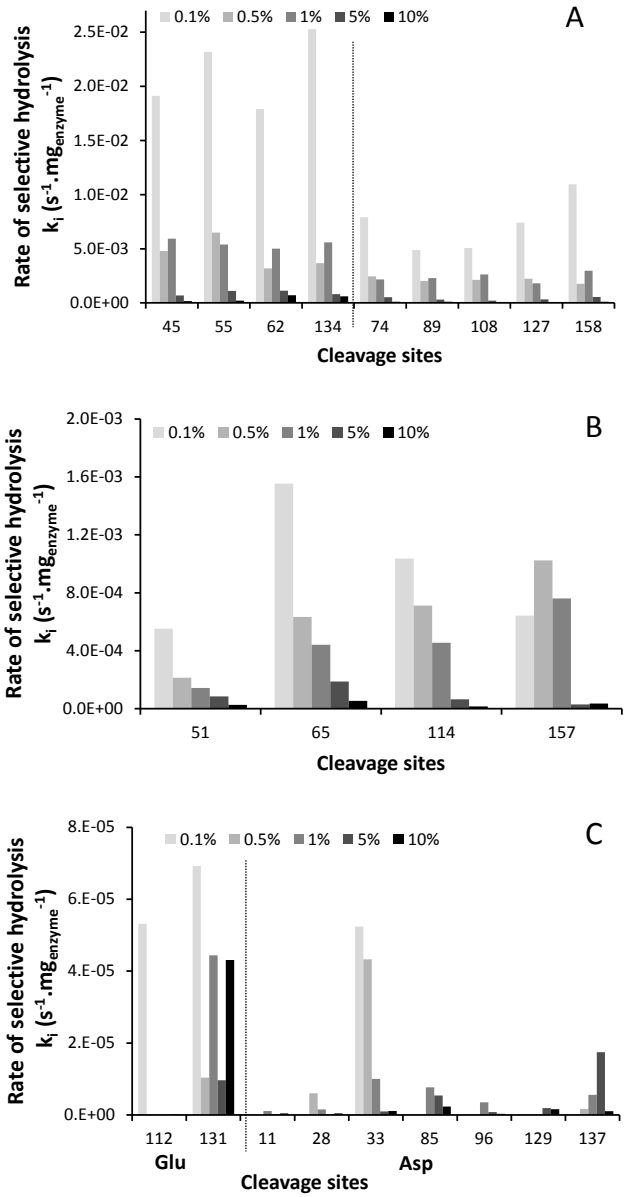


Figure 6. Rate of selective hydrolysis k_i of the enzyme towards glutamic acid and aspartic acid residues divided in three groups with (A) Glu with high and intermediate rate of selective hydrolysis and (B) Glu with low rate of selective hydrolysis and (C) Glu with very low rate of selective hydrolysis and Asp for the different initial protein concentrations (0.1-10 % (w/v) WPI).

The third group consists of four Glu residues for which the rate of selective hydrolysis is 10 times lower than the first two groups, $4.3 \cdot 10^{-4}$ $s^{-1} \cdot mg^{-1}_{enzyme}$. (**figure 6B**) Two Glu residues have a rate comparable to the Asp residues ($9.5 \cdot 10^{-6}$ $s^{-1} \cdot mg^{-1}_{enzyme}$) forming a

fourth group. (**figure 6C**) Finally one Glu residue (44) and four Asp residues (53, (A)-64, 98 and 130) are not hydrolyzed at all, constituting the fifth group.
While the overall division of the cleavage sites into 5 groups is comparable for all initial substrate concentrations, for a few of these cleavage sites a clear effect of initial substrate concentration is observed. For the nine Glu residues for which the enzyme has a very high or high rate of selective hydrolysis, a decrease in the rate of selective hydrolysis is observed with increasing substrate concentration (from $1.3 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ for 0.1 % (w/v) WPI to $2.3 \cdot 10^{-4}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ for 10 % (w/v) WPI. (**figure 6A**) This was expected, since the overall hydrolysis rate determined from the DH versus time curves was decreased. (**figure 1B**)

Non-annotated cleavage sites

For five out of 27 potential cleavage sites, no rate of hydrolysis was determined, because no peptides were annotated starting or ending at these cleavage sites. There are two reasons for this. First, cleavage sites 53-D and 130-D are surrounded by other cleavage sites (51-E, 55-E and 129-D, 131-E, respectively), for which cleavages were observed. The cleavages occurring on 53-D and 130-D might lead to the formation of single amino acids or di-peptides. The short peptides and free amino acids are generally not detected during LC-MS analysis. The difficulty to annotate short peptides and free amino acids in RP-HPLC has been mentioned before [14]. This could be solved by analyzing the free amino acids, using a different gradient or a different type of column than reversed phase for instance. Secondly, for cleavage sites 44-E and 64-E, a glutamic acid on the P1' position is found: 45-E and 65-E. It has been previously suggested that the presence of a negative charge on position P1' hinders the hydrolysis by BLP [9].

Determination of the selectivity

The selectivity was determined for all cleavage sites identified for all protein concentrations (0.1-10 % (w/v) WPI). The selectivity is determined as the rate of selective hydrolysis of each cleavage site divided by the rate of selective hydrolysis of all cleavage sites for each substrate concentration. (equation 8, **table 2**) Large differences are observed over all cleavage sites for each initial substrate concentrations from 17 to 0.001 %. The cleavage sites are divided into five groups, based on the groups defined for the rate of selective hydrolysis. (**figure 6**) The enzyme selectivity towards the first four Glu residues is on average ~ 16 %. The selectivity towards the second group (5 Glu) is ~ 6 % and towards the third group (4 Glu) the average selectivity is ~ 1.4 %. The fourth group contains 2 Glu and 7 Asp, for which the enzyme has a selectivity of ~ 0.027 %. The fifth group contains the cleavage sites for which no hydrolysis was observed. (**table 2**) Enzyme selectivity has never been studied. There are consequently no studies available to compare the findings described in this study.

Table 2. Selectivity$_{\beta\text{-lg}}$ (%) of the enzyme towards all cleavage sites for the different initial protein concentration (0.1-10 % (w/v) WPI).

	Cleavage site	0.1 %	0.5 %	1 %	5 %	10 %
Glu with high selectivity	45	15	15	17	11	7
	55	18	21	15	18	9
	62	14	10	14	19	30
	134	20	12	16	13	26
Glu with intermediate selectivity	74	6	8	6	9	5
	89	4	6	6	5	5
	108	4	7	7	3	1
	127	6	7	5	5	3
	158	9	6	8	9	5
Glu with low selectivity	51	0.44	0.68	0.40	1.4	1.1
	65	1.2	2.0	1.2	3.1	2.3
	114	0.82	2.3	1.3	1.1	0.7
	157	0.51	3.3	2.1	0.5	1.6
Glu with very low selectivity - Asp residues	112	0.042	n.d.	n.d.	n.d.	n.d.
	131	0.055	0.0083	0.035	0.0077	0.034
	11	n.d.	n.d.	0.0031	0.0036	0.018
	28	n.d.	0.019	0.0044	0.0017	0.019
	33	0.042	0.14	0.028	0.016	0.048
	85	n.d.	n.d.	0.022	0.090	0.10
	96	n.d.	n.d.	0.0099	0.013	0.015
	129	n.d.	n.d.	n.d.	0.032	0.071
	137	n.d.	0.0054	0.016	0.29	0.047
Non-hydrolyzed Glu and Asp residues	44	n.d.	n.d.	n.d.	n.d.	n.d.
	53	n.d.	n.d.	n.d.	n.d.	n.d.
	A-64	n.d.	n.d.	n.d.	n.d.	n.d.
	98	n.d.	n.d.	n.d.	n.d.	n.d.
	130	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: Peptides were analyzed until a DH of 7 %, for certain cleavage sites no corresponding peptides were found, indicating that the selectivity is < 0.001 %.
Note: The sum of selectivities of all cleavage sites at each substrate concentration is 100 %.

Influence of substrate concentration

The large differences in selectivity towards all cleavage sites is quite similar for all substrate concentrations. Still, two main effects of protein concentration are observed on the selectivity, firstly in the group with the highest selectivity and secondly on the group with aspartic acid residues. First, in the group of cleavage sites for which the enzyme has the highest selectivity, the selectivity towards cleavage sites 62 and 134 is two times higher at 10 % (w/v) WPI than at the other protein concentrations. (**figure 7**) The selectivity towards 45 and 55 on the other hand is two times lower at 10 % (w/v) WPI than at the other concentrations. (**figure 7**) It should be noted that there is a small decrease in the total UV annotated at increasing DH. Hence care must be taken in data interpretation. For other cleavage sites after Glu residues, some influence of the concentration are observed. For instance, cleavage site 51 has a higher selectivity at high initial substrate concentrations (5 and 10 % (w/v) WPI) than at low substrate concentrations (0.1-1 % (w/v) WPI). Cleavage sites, such as 51 have a lower selectivity than cleavage sites 45, 55, 62 and 134. Consequently their influence is less on the mechanism of hydrolysis.

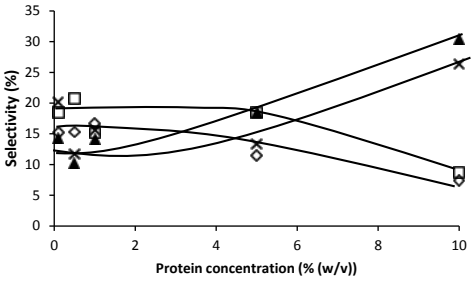


Figure 7. Enzyme selectivity towards four cleavage sites (◊) 45, (□) 55, (▲) 62 and (**x**) (134) as a function of the initial substrate concentration.

Secondly, the enzyme selectivity towards the cleavage sites after Asp residues, is higher at high initial substrate concentrations (> 1 % (w/v)) than at the low concentrations (0.1-1 % (w/v) WPI). The average selectivity towards Asp residues is 0.01 % for low concentrations (0.1-1 % (w/v) WPI) and 0.06 % for high concentrations (5 and 10 % (w/v) WPI). (**table 2**)

Conclusions

By increasing the substrate concentration during enzymatic protein hydrolysis, the overall hydrolysis rate is decreased and a lower DH is reached. For the cleavage sites (Glu-62 and Glu-134) and (Glu-45 and Glu-55), for which the enzyme has a very high selectivity, a two times higher or lower selectivity, respectively, is observed at 10 % (w/v) WPI than at the other substrate concentrations. In addition, the enzyme selectivity towards Asp residues is higher at high initial substrate concentrations (≥ 5 % (w/v) WPI) than at low substrate concentrations. This shows that changing the substrate concentration influences both the overall hydrolysis rate and the mechanism of hydrolysis.

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Chapter 6

Influence of the pH of hydrolysis on the enzyme selectivity

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Abstract

The enzymatic hydrolysis of proteins is typically described by the degree of hydrolysis and by the enzyme specificity. While the specificity describes which cleavage sites can potentially be cleaved, it does not describe which cleavage sites are used. To identify the relative rate at which each individual cleavage site is hydrolyzed, the enzyme selectivity has recently been introduced. Large differences were observed in the selectivity towards different cleavage sites. This opened the possibility to study the changes in selectivity that result from changes in system conditions such as the pH of hydrolysis. Since enzymes are typically characterized by their pH optimum, based on total activity, it was decided to test if the selectivity was affected by the pH. A 1 % whey protein isolate (WPI) was hydrolyzed by BLP (*Bacillus licheniformis* protease) at pH 7, 8 and 9. The selectivity was determined from identification and quantification of all peptides (by UPLC-MS) formed at different degrees of hydrolysis. For each hydrolysate, up to 90-95 % of the UV_{214} peak area was attributed to peptides. At all pH values, large differences in the enzyme selectivity (from < 0.004 % to 15 %) towards the different cleavage sites of β-lactoglobulin were observed. The increase in pH had different effects on the selectivity towards different cleavage sites. For some cleavage sites, the selectivity increased with pH, while others decreased, and still others showed a maximum, or a minimum at pH 8.0. Three cleavage sites (44, 129 and 157), which have a residue with a negatively charged side chain on P1' show a higher selectivity at pH 7.0 than at pH 8.0 and 9.0. This trend seems to correlate with changes in the charge state of the side chain of the His residue present in the active site of the enzyme. However, for the other cleavage sites no clear correlation was found between the primary structure of the substrate and the change in selectivity as a function of pH. The significant variation in selectivity shows that this parameter may be useful in understanding the peptide release kinetics in enzymatic hydrolysis under different conditions.

Submitted

Introduction

While enzymatic hydrolysis of proteins is often studied, little is known about the mechanism of hydrolysis. The characterization of protease activity, for instance, often starts by identifying the optimal pH and temperature for the hydrolysis. The optimum is then defined as the condition at which the highest conversion after a set amount of time is observed. In this way, the optimal pH of BLP (*Bacillus licheniformis* protease) was determined to be pH 8.0 [1]. The activity can also be determined from the rate of protein hydrolysis using plots of the degree of hydrolysis (DH) vs. time. In this way, an increase in activity of a protease from *Bacillus subtilisis* with increasing pH (from 8 to 10) towards the hydrolysis of whey proteins was determined [2]. Neither method, however, yields information on the mechanism of hydrolysis or on the peptides that are released.

A first indication of the mechanism of hydrolysis is obtained from the decrease of the amount of intact protein as a function of the DH. It has, for instance, been shown that during hydrolysis of haemoglobin by pepsin the proportion of remaining intact protein (at any DH) was higher at pH 4.5 than pH 3.5 [3]. A slow degradation of the intact protein (as a function of DH) implies that the enzyme has more difficulty in hydrolyzing the intact protein than in hydrolyzing the intermediate peptides formed. On the other hand a fast degradation of the intact protein indicates that no such hindrance is present. These two extreme cases have been referred to as one-by-one and zipper mechanism, respectively [4].

The accessibility of the intact protein is important, but still does not contain information on which peptide will be released preferentially. For such information, the selectivity of the enzyme towards the different cleavage sites needs to be determined. The selectivity was recently defined as the relative rate at which the enzyme cleaves each individual cleavage site compared to the total rate of hydrolysis of all cleavage sites in the protein (chapter 4). In this way, the selectivity is more detailed than the enzyme specificity, which only describes the amino acids after which an enzyme will cleave a peptide bond. An indication that the pH of hydrolysis influences the enzyme selectivity was found in the tryptic hydrolysis of β-casein [5]. It was shown that the kinetics of peptide formation and release of peptides with Arg residues were not affected by the changes in pH, while for peptides after Lys the pH of hydrolysis did influence the peptide kinetics. In this example, the change in selectivity is explained by changes in the charge state of the amino acid on the cleavage site. For other enzymes, the changes in selectivity may be due to charges of amino acids in the neighborhood of the cleavage site. Moreover, changes in pH can also affect the charge state of amino acids in the active site of the enzyme, although it is currently not well understood if this would indeed change the enzyme selectivity.

To determine the influence of the pH on the selectivity of the enzyme, whey protein isolate (WPI) was hydrolyzed by BLP at different pH values. In addition to the selectivity, the hydrolysis was characterized by three other descriptors: (1) the rate of hydrolysis, (2) the proportion of remaining intact β-lactoglobulin as a function of the DH

and (3) the peptide molecular weight distribution. For the latter, the peptides obtained at the different DH values were annotated and quantified. The selectivity was then determined from the change in concentration of each individual peptide with time, peptide release kinetics.

Materials and Methods

Materials. Bipro, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). The provided WPI contained (by weight) 74.0 % β-lactoglobulin, 12.5 % α-lactalbumin, 5.5 % bovine serum albumin and 5.5 % immunoglobulin, according to specifications of the supplier. The protein content (Nx6.32) [6] of the powder was 93.4 % (w/w) as determined by Dumas. BLP (*Bacillus licheniformis* protease), specific for Glu-X bonds and for Asp-X bonds [7], was obtained from Novozymes (NS-37005) (Bagsvaerd, Denmark). BLP (4.5 % (w/w) protein, Nx6.25, 0.3 AU/mg/min as determined by azocasein assay [8]) was partly insoluble and was fractionated by first solubilizing the BLP powder in 10 mM NaH_2PO_4 pH 5.8 and stirred overnight at 4 °C. The suspension was centrifuged (10 min, 4000 g, 25 °C). The supernatant obtained was extensively dialyzed against 150 mM NaCl, followed by dialysis against demineralized water using cellulose dialysis membranes (cut-off 12-14 kDa). The freeze dried material was found to contain 60 % (w/w) protein (Nx6.25) from which 78 % is the enzyme and 14 % is the pro-peptide (6.9 kDa) as determined previously (chapter 3). An activity of 3.9 AU/mg/min was determined by the azocasein assay. All other chemicals were of analytical grade and purchased from Sigma or Merck.

Hydrolysis

Protein solutions were prepared by dispersing WPI powder at a concentration of 45 % (w/v) in Millipore water, followed by stirring overnight at 4 °C. Insoluble parts were removed by centrifugation (30 min, 4000 g, 20 °C) and the supernatant obtained (30 % (w/v)) was diluted to 1 % (w/v) as determined by UV_{280} [6]. The 1 % (w/v) protein solutions were obtained from separate dispersions. Hydrolyses were performed using a pH-stat. Solutions of WPI at 1 % (w/v) were hydrolyzed at 40 °C and pH 7.0, 8.0 or 9.0 using 0.2 M NaOH to keep the pH constant. Protein solutions (10 mL) were equilibrated at least 15 minutes at 40 °C and adjusted to pH 7.0, 8.0 or 9.0 before addition of BLP dissolved at 5 % (w/v) in Millipore water (0.30 µL of enzyme/ mg of protein). The degree of hydrolysis (DH) was calculated based on the added volume of NaOH using $1/\alpha$ = 1.20 at 40 °C and pH 8.0; 3 at pH 7 and 1.02 at pH 9 and h_{tot}(WPI) = 8.5 as described before ([6], chapter 3).

Samples were taken during the hydrolysis at different degrees of hydrolysis (1.5, 3, 4.5, 6 and 7 %). The samples were first centrifuged (5 min, 19000 g, 15 °C). Supernatant and pellet were separated, and the supernatants were inactivated by adjusting the pH to 2 with 5 M HCl. The pH was readjusted to 8.0 with NaOH after at least 10 minutes of inactivation before storage of the samples at -20 °C.

The initial rate of hydrolysis of BLP towards WPI was determined from the slope of the linear portion of the DH vs time curves as described previously (chapter 3). The initial rate of hydrolysis is expressed here as the concentration of bonds cleaved per mg of enzyme per time in seconds ($mol \cdot L^{-1}_{bonds} \cdot s^{-1} \cdot mg_{enz}^{-1}$).

Reverse Phase Ultra High Performance liquid chromatography (RP-UHPLC)

Samples obtained during hydrolysis were analyzed on an Acquity UPLC System (Waters, Milford, MA, USA) using an Acquity UPLC BEH 300 C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 x 50 mm, 1.7 µm particle size). Eluent A was 1 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) in Millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. To reduce disulfide bridges and to facilitate peptide annotation, samples were first incubated for two hours with 100 mM dithiothreitol (DTT) in 50 mM Tris·HCl, pH 8.0 at a concentration of 0.5 % (w/v). After incubation, samples were further diluted in eluent A to a final concentration of 0.1% (w/v) and centrifuged (10 min, 19000 g, 20 °C). Samples (4 µL) were injected into the column thermostated at 40 °C (chapter 4).

The amount of remaining β-lactoglobulin (A + B) and α-lactalbumin was determined using the following elution profile: 0-2 min isocratic on 30 % B; 2-12 min linear gradient from 30 % B to 50 % B; 12-15 min linear gradient from 50 to 100 % B; 15-20 min isocratic on 100% B; 20-21 min from 100 % B to 30 % B and 21-30 min isocratic on 30 % B.

For separation of the peptides a different elution profile was used: 0-2 min isocratic on 3 % B; 2-10 min linear gradient from 3 % to 22 % B; 10-16 min linear gradient 22 - 30 % B; 16-19 min linear gradient 30-100 % B; 19-24 min isocratic on 100 % B; 24-26 min linear gradient 100-3 % B and 26-30 min isocratic on 3 % B. The flow rate was 350 $\mu L \cdot min^{-1}$. Detection was performed using a PDA, which was scanning the absorbance from 200-400 nm at a 1.2 nm resolution, with 20 points per second.

Electron spray ionization time of flight mass spectrometry (ESI-Q-TOF-MS)

The mass spectra of the hydrolysates were determined with an online Synapt high definition mass spectrometer (Waters), coupled to the RP-UPLC system, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). The system was calibrated using Glu-1-Fib. The capillary voltage was set to 3 kV with the source operation in positive ion mode and the source temperature at 120 °C. The sample cone was operated at 35 V. Nitrogen was used as desolvation gas (250 °C, 800 L/h) and cone gas (200 L/h). The trap gas was set at 1.5 mL/min. MS and MS/MS (MSe method) were performed between m/z 100-2000 with a scan time of 0.3 seconds. The trap collision energy was set at 6 V in single MS mode and ramped from 20 to 30 V in MSe mode. The transfer collision energy was set at 4 V in MS and switched between 4 and 10 V in the MSe mode. UV and MS data were acquired using MassLynx software v4.1 (Waters).

Peptide identification and quantification

The proportion of remaining intact α-lactalbumin and β-lactoglobulin (A and B) was determined by dividing the area of the UV_{214} peak obtained for each protein at different DH of hydrolysis by the peak area obtained for each intact protein before hydrolysis.

For peptide identification, internal lock mass was applied on every chromatogram for MS and MS/MS data with two previously identified masses chosen among the most abundant masses, of which the sequences were identified manually. The identification of MS and MSe ion peaks was done by Biopharmalynx 1.3 software with the parameters described before (chapter 4). The annotation was first performed using a method which identifies peptides resulting from cleavages only after Glu and Asp. Subsequently, a generic method was used where no enzyme specificity was selected. For UV peaks where the automatic annotation did not yield a peptide, a manual analysis of the MS and MS/MS data was used to assign the peptide. For the total peptide quantification, peaks in the UV_{214} chromatogram were included until 90-95 % of the total UV_{214} area was included. Peptides from β-lactoglobulin and α-lactalbumin as well as the intact protein were quantified using the UV_{214}. In case of co-eluting peptides from either β-lactoglobulin or α-lactalbumin, the UV peak area was divided over the co-eluting peptides in a ratio based on the MS intensity (chapter 4). Only peptides from β-lactoglobulin were considered for the determination of the selectivity.

The quantification of the peptides was based on the UV signal at 214 nm, using equation (1):

(1) $$C_{peptide} = 1 * 10^6 \left(\frac{A_{214}}{\varepsilon_{214} l V_{inj} k_{cell}}\right) Q$$

In which $C_{peptide}$ (µM) is the concentration of peptide, A_{214} (AU.min) is the UV peak area at 214 nm, V_{inj} (µL) is the volume of sample injected, Q the flow rate in $\mu L \cdot min^{-1}$, l is the path length of the UV cell, which is 1 cm according to the manufacturer. The value for the cell constant k_{cell} was previously determined to be 0.66 using pure peptides, but depends on the geometry of the UV detector used [9]. The molar extinction coefficient at 214 nm of each peptide (ε_{214}) was calculated as described before [10].

Molecular mass distribution profile

The weight-based concentration of each peptide resulting from hydrolysis of β-lactoglobulin was calculated from the molar concentration. The peptides were divided into molecular mass ranges (<1 kDa, 1-1.5 kDa, 1.5-2 kDa, 2-2.5 kDa, 2.5-3 kDa, > 3 kDa and intact protein). The weight-based concentrations of the different peptides were summed in each molecular mass range.

Quality of the annotation and of the quantification

The quality of the peptide identification and of the quantification was described by three different parameters. First, the amino sequence coverage was determined by calculating the number (#) of unique annotated amino acid divided by the total number (#) of amino acids present in the parental protein sequence:

(2) $$Amino\ acid\ sequence\ coverage = \frac{\#\ unique\ annotated\ amino\ acids}{\#\ amino\ acids\ in\ protein\ sequence}$$

This was found to be 100 % for all samples. To take into account the fact that each part of the sequence is covered by different peptides, the peptide sequence coverage was determined as a second quality parameter on the peptide annotation (chapter 4). It is defined as the number of amino acids (#AA) in annotated peptides divided by the total number of amino acids (#AA) present in all peptides (annotated peptides and missing peptides) as in equation (3)

(3) $$Peptide\ sequence\ coverage = \frac{\#\ AA\ (annotated\ peptides)}{\#\ AA(annotated\ peptides)+\#\ AA\ (missing\ peptides)}$$

The peptide sequence coverage was calculated to be 96 ± 3 % on average. To determine the completeness of the quantification, the molar sequence coverage is calculated. For that, the concentration of each amino acid C_n was calculated using the molar concentration of each individual (annotated) peptide $C_{peptide}$. With this, each individual amino acid of the primary sequence of β-lactoglobulin has been quantified in the hydrolysate. The molar sequence coverage is calculated using equation (4):

(4) $$Molar\ sequence\ coverage(\%) = (1 - \frac{\sqrt{\frac{\sum(C_n - C_0)^2}{(\#AA-1)}}}{C_0}) \times 100$$

Where C_n (μM) is the concentration of each individual amino acid n in the protein obtained from quantification of the peptides; C_0 (μM) is the initial concentration of the protein and #AA is the number of amino acids in the sequence of the parental protein (chapter 4).

In addition, the degree of hydrolysis (DH) was calculated from the molar quantification of all peptides using equation (5)

(5) $$DH\ (\%) = \frac{\sum C_{peptides} - C_0}{C_o \times \#_{bonds} - C_o} \times 100$$

In which $C_{peptide}$ and C_0 are the concentrations of all peptides in the hydrolysates and the initial concentration of protein (μM), respectively. $\#_{bonds}$ is the number of bonds in the protein.

Determination of the enzyme selectivity

To describe the selectivity of the enzyme, the rate at which individual peptide bonds are hydrolyzed (e.g. between Glu-45 and Leu-46), was determined. To do this, first the concentrations of all peptides originating from hydrolysis of the same peptide bond ($C_{i,t}$) in the parent molecule are calculated using equation (6)

(6) $$C_{i,t} = \sum \left\{ C_{peptide}\left[x-y\right]_t \mid i = x-1 \bigcup i = y \right\}$$

In other words, the concentration of cleavage products formed at each time point t, after hydrolyzing peptide bond no. i equals the sum of all peptides of sequence [x-y], for which i = (x-1) or i = y. This means it is the sum of the peptides that are formed from hydrolysis after the amino acid i or which end by the amino acid i.

For each cleavage site the apparent cleavage rate ($k_{i,app}$ in s^{-1}) was then calculated by fitting equation (7) to the experimental data.

(7) $$C_{i,t} = C_0 - C_0 e^{-k_{i,app} \times t}$$

In which $C_{i,t}$ is the concentration of cleavage products (μM) determined for each time point t and C_0 is the initial concentration of protein (μM).

The rate of selective hydrolysis k_i ($s^{-1} \cdot mg^{-1}_{enzyme}$) is defined as:

(8) $$k_i = \frac{k_{i,app}}{m_E}$$

in which m_E is the mass of enzyme added for the hydrolysis (in mg). Subsequently, the $selectivity_{\beta\text{-lg}}$ (%) is calculated by dividing the rate of selective hydrolysis k_i of each individual cleavage site by the sum of the rate of selective hydrolysis of all cleavage sites at each protein concentration as in equation (9):

(9) $$Selectivity_{\beta-lg}\ (\%) = \frac{k_i}{\sum k_j} \times 100$$

The relative selectivity was calculated using the highest selectivity for each cleavage site as 100 %.

Reproducibility of annotation and quantification

To test the reproducibility of the quantification method, one sample (1 % WPI, DH 4.5 %) was injected 3 times. The concentration determined for individual peptides showed a typical standard error of 6 %. In addition, the reproducibility of the complete analysis was tested. For this, two separate hydrolysis experiments were performed at the same protein concentration (0.5 % (w/v) WPI). During each hydrolysis, samples were taken at DH 1.5, 3, and 6 %. Peptides were annotated and quantified for all hydrolysates. The error in the sequence coverage was calculated for the hydrolysates at each DH. The values of errors were averaged to determine the analysis error. The quality of the annotation as described by the peptide sequence coverage showed a standard error of 2.5 %. The molar sequence coverage showed a standard error of 18 %. The determined selectivity showed a typical standard error of 15 %.

Results and Discussion

Protein hydrolysis at different pH values

The initial rate of hydrolysis of a 1 % whey protein isolate (WPI) by BLP, calculated from the initial part of the DH vs time curves is 2 times higher at pH 9 than at pH 7. (**figure 1A**) In addition, by increasing the pH from 7 to 9, an increase of the DH reached at 10,000 s of hydrolysis is observed, with DH = 4 % reached at pH 7 and DH = 8 % at pH 9. (**figure 1B**) These results agree with reported activity measurements of BLP towards whey protein concentrate [11]. However, as mentioned in the introduction, an optimum activity of BLP at pH 8 has also been described [1]. In that study the activity showed a bell-shaped curve with an optimum at pH 8 and lower activities at pH 7 and 9. Since this was determined from the release of the fluorescent part of a synthetic

peptide, and not on WPI, it is concluded that such observed effects depend on the substrate used.

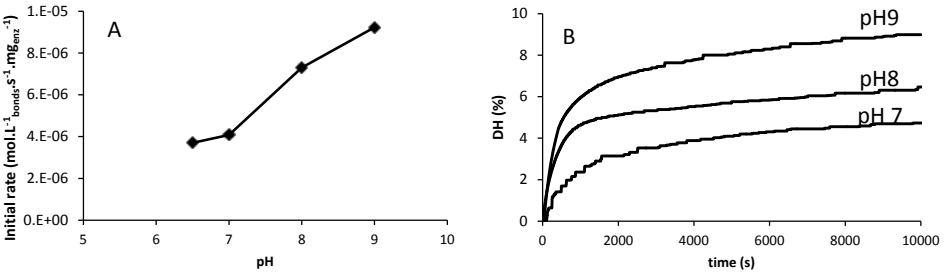


Figure 1. (A) Initial rate of hydrolysis ($mol \cdot L^{-1}_{bonds} \cdot s^{-1} \cdot mg_{enz}^{-1}$) of the enzyme BLP and (B) hydrolysis curves of 1 % WPI hydrolyzed by BLP at different pH and at 40°C.

In addition to a change in the initial rate and DH reached, a change in activity towards intact protein is observed. The proportion of remaining intact β-lactoglobulin at any DH increased in the order pH 7 > pH 8 > pH 9. (**figure 2A**) For example, at DH = 3 % there is 10 % remaining β-lactoglobulin at pH 9, 20 % at pH 8 and 50 % at pH 7. The affinity of the enzyme for the intact protein (compared to the affinity for derived peptides) can be estimated from the slope of the linear decrease of intact β-lactoglobulin. The affinity of BLP towards β-lactoglobulin is 3 times higher at pH 9 than at pH 7. A similar effect was observed for the hydrolysis of WPI by trypsin, where the concentration of intact β-lactoglobulin was decreased faster at pH 8.5 than at pH 7 (150 $mg \cdot min^{-1}$ at pH 8.5 and 50 $mg \cdot min^{-1}$ at pH 7) [12].

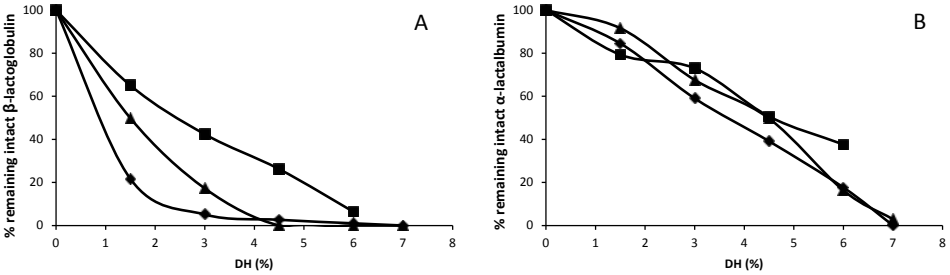


Figure 2. Proportion of (A) remaining β-lactoglobulin and (B) remaining α-lactalbumin as a function of DH determined by pH-stat, for the different pH of hydrolysis (■) pH 7, (▲) pH 8 and (♦) pH 9.

This change in activity towards intact proteins may be related to the stability of the protein. It has been suggested that the rate of hydrolysis increases with the decrease in thermal stability [13]. The stability of β-lactoglobulin, determined by the denaturation temperature decreases from 76 °C at pH 7 to 58 °C at pH 9 as determined by DSC at

100 mM NaCl [14]. This is an indication that the structural stability of proteins is closely related to their susceptibility to enzymatic hydrolysis.
For α-lactalbumin, in contrast to β-lactoglobulin, the data for all pH values fall on the same curve. (**figure 2B**) The proportion of remaining intact α-lactalbumin decreases almost linearly with the DH. This shows a low affinity of the BLP for the intact α-lactalbumin. The similar degradation profiles of the intact α-lactalbumin correlates with the fact that the stability of α-lactalbumin does not change as a function of pH as shown by DSC [15]. β-Lactoglobulin is preferred over α-lactalbumin as expected based on the higher proportion of β-lactoglobulin than α-lactalbumin in the WPI used. Furthermore, β-lactoglobulin contains relatively more Glu residues than α-lactalbumin. The lower concentration of α-lactalbumin and the lower content of Glu residues result in a lower rate of hydrolysis of this protein compared to β-lactoglobulin.

Identification and quantification of the peptides

Only peptides resulting from hydrolysis of β-lactoglobulin are analyzed in detail to determine the influence of pH on the mechanism of hydrolysis. In total by combining all hydrolysates, 84 peptides resulting from hydrolysis of β-lactoglobulin were annotated and quantified. (**Annexes 2 and 4**) While most formed peptides agreed with the known specificity of BLP (i.e. for Glu and Asp), a total of 16 a-specific peptides were annotated.
The quality of the quantification, described by the molar sequence coverage was found to be on average 76 ± 10 % over all hydrolysates. (equation 4) To further describe the quality of the quantification, the weight-based concentration of each peptide was calculated and summed at each DH. (**table 1**) The total weight based concentration decreases with increasing DH. This is in part explained by the decrease in total area annotated. Still, a decrease in the total UV_{214} area is expected at increasing DH due to a decrease in the number of peptide bonds. In addition, some losses in the sample preparation due to lower solubility of certain peptides in the eluent might also result in a decrease of the total UV. The proportion of UV area attributed to β-lactoglobulin (72 % on average) is quite similar for all samples. This proportion corresponds to the proportion of β-lactoglobulin in the WPI used (74 %). The variation in the concentration of β-lactoglobulin at DH = 0 % for the three pH of hydrolysis is due to daily variations in the sample preparation. Based on the molar concentration of peptide and intact protein concentrations determined during analysis of each hydrolysate, the degree of hydrolysis of β-lactoglobulin was calculated. (equation 5) It was found that the calculated DH values are slightly underestimated compared to the values obtained with the pH-stat at high DH. (**table 1**) This can be partly explained by the fact that only peptides from β-lactoglobulin are included in this calculation of the DH, while the value calculated by the pH-stat represents the hydrolysis of all proteins in WPI.
The concentration of the 16 a-specific peptides increases with DH and with decreasing pH. (**table 1**) The increase in concentration is thus correlated to increasing time

incubation rather than conditions of hydrolysis. The overall hydrolysis rate is indeed slower at decreasing pH.

Table 1. Total concentration of the peptides annotated as a function of DH for the three pH of hydrolysis.

pH 7	DH (%)				
	0	1.5	3	4.5	6
Concentration intact β-lactoglobulin (mg/L)	647	540	352	275	48
Concentration specific peptides (mg/L)		165	290	381	332
Concentration a-specific peptides (mg/L)		0	2.8	22.6	98.6
Total concentration (mg/L)	647	705	645	678	479
Total concentration (µmol/L)	35	101	172	263	343
Calculated DH (β-lg) (%)	0	1.2	2.4	4.1	5.5
Annotated UV area (AU.min)	98850	99318	89236	85358	76513
Annotated β-lactoglobulin UV area (AU.min)	78181	83803	74329	64957	52789

pH 8	DH (%)					
	0	1.5	3	4.5	6	7
Concentration intact β-lactoglobulin (mg/L)	556	313	66	0	0	0
Concentration specific peptides (mg/L)		316	430	420	330	272
Concentration a-specific peptides (mg/L)		0	1.3	3.0	25.6	51.7
Total concentration (mg/L)	556	629	498	423	355	324
Total concentration (µmol/L)	30	156	230	290	303	314
Calculated DH (β-lg) (%)	0	2.2	3.5	4.6	4.8	5.0
Annotated UV area (AU.min)	80005	93680	76297	73438	65984	55619
Annotated β-lactoglobulin UV area (AU.min)	67126	72957	52640	50632	43988	36168

pH 9	DH (%)					
	0	1.5	3	4.5	6	7
Concentration intact β-lactoglobulin (mg/L)	582	159	21	0	0	0
Concentration specific peptides (mg/L)		311	441	520	486	460
Concentration a-specific peptides (mg/L)		0	1.0	4.9	21.8	29.9
Total concentration (mg/L)	582	471	463	525	507	490
Total concentration (µmol/L)	32	126	217	298	355	391
Calculated DH (β-lg) (%)	0	1.6	3.3	4.7	5.7	6.4
Annotated UV area (AU.min)	93808	78910	76919	79923	76968	78200
Annotated β-lactoglobulin UV area (AU.min)	70334	54748	52133	57688	55798	53230

As an indication of changes in the peptide profile due to changes in the pH of hydrolysis, the weight-based concentration of all peptides was summed in each molecular mass class. (**figure 3**) At DH = 3 %, less intact β-lactoglobulin and a larger amount of intermediate peptides (> 3000 Da) is found at pH 8 and 9 than at pH 7. (**figure 3A**) At DH = 6 %, only at pH 7 a small amount of intact β-lactoglobulin is still present. This resulted in a lower quantity of peptides in the mass range from 1500 to 3000 Da at pH 7 than at pH 8 and pH 9. (**figure 3B**)

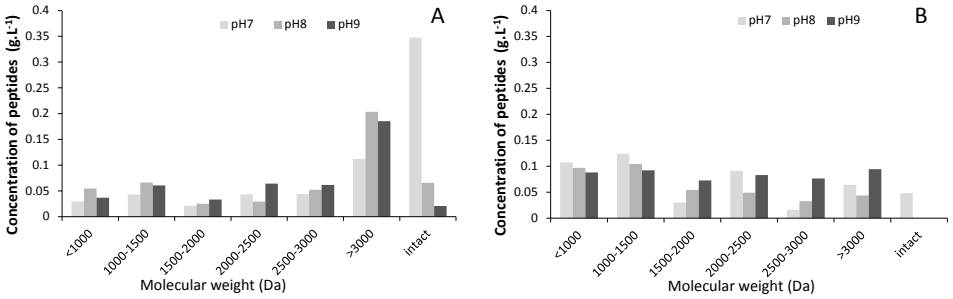
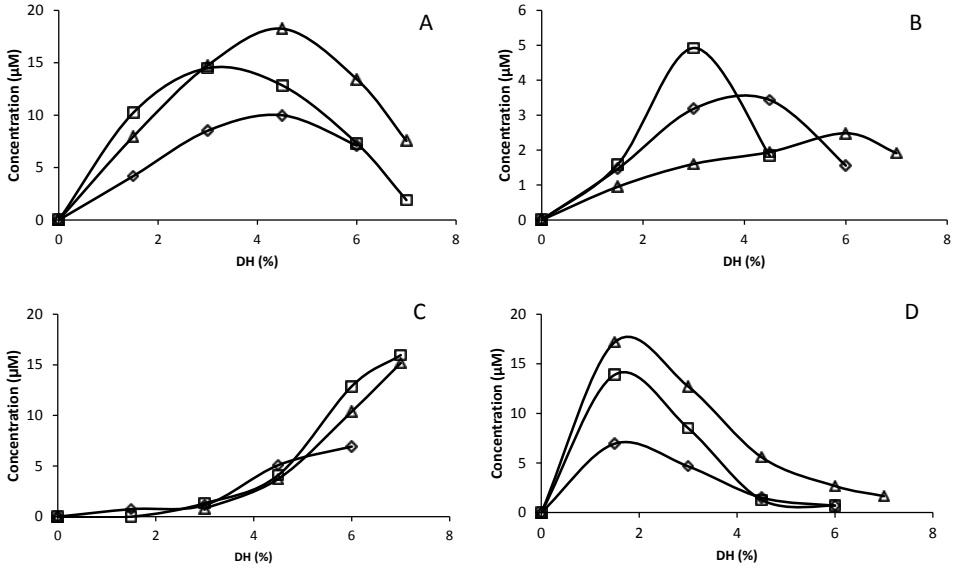


Figure 3. Molecular weight distribution for three different pH of hydrolysis for (A) DH = 3 % and (B) DH = 6 % of 1 % WPI hydrolyzed by BLP.

Influence of the pH on the enzyme selectivity

Kinetics of peptide formation

To characterize the influence of the pH of hydrolysis on the mechanism of hydrolysis, first the rate of formation and further breakdown of the peptides as a function of the DH for the different pH of hydrolysis were determined. Four peptides representative of the different types of peptide release kinetics are shown. (**figure 4**)



(A)	β-lg[1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE
(B)	β-lg(A)[90-127]	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPE
(C)	β-lg(A)[115-127]	(E)-QSLVCQCLVRTPE
(D)	β-lg[135-162]	(E)-KFDKALKALPMHIRLSFNPTQLEEQCHI

Figure 4. Peptide concentration as a function of the degree of hydrolysis (DH) for (◊) pH 7, (□) pH 8 and (Δ) pH 9. All hydrolysates were diluted to 0.1 % (w/v) prior to analysis.

As described in a previous study, peptides β-lg[1-45] and β-lg[135-162] are intermediate peptides that can be formed from one cleavage on the intact protein.

Peptide β-lg(A)[90-127] is an intermediate peptide that can be formed from two cleavages on the intact protein. Peptide β-lg(A)[115-127] is a final peptide (chapter 4). The concentration of the peptides as a function of the DH shows comparable trends for each pH, while different maximum concentrations are reached. For instance, the maximum concentration of peptide β-lg[1-45] is 18 µM at pH 9; 14 µM at pH 8 and 10 µM at pH 7, so the maximum concentration is 50 % lower at pH 7 than at pH 9. (**figure 4A**) Similarly, for peptide β-lg[135-162], a difference of 50 % is observed in the maximum concentration of the peptide at pH 7 and 9. (**figure 4D**) The concentration of peptide β-lg-(A)[115-127], a final peptide, increases with increasing DH in the same way for all pH. (**figure 4C**) This shows significant differences in the mechanism of hydrolysis as a function of pH. For some peptides there is a larger accumulation of intermediate peptides at pH 9 than at pH 7. For other peptides, the change in pH has no significant effect.

Kinetics of the cleavage site products formation

After annotation and quantification of all peptides, the concentration of cleavage products $C_{i,t}$ is calculated using equation 6 to determine the apparent cleavage rate $k_{i,app}$. (equation 7) The concentration of cleavage products $C_{i,t}$ is compared for the three pH values of hydrolysis for four typical examples. (**figure 5**)

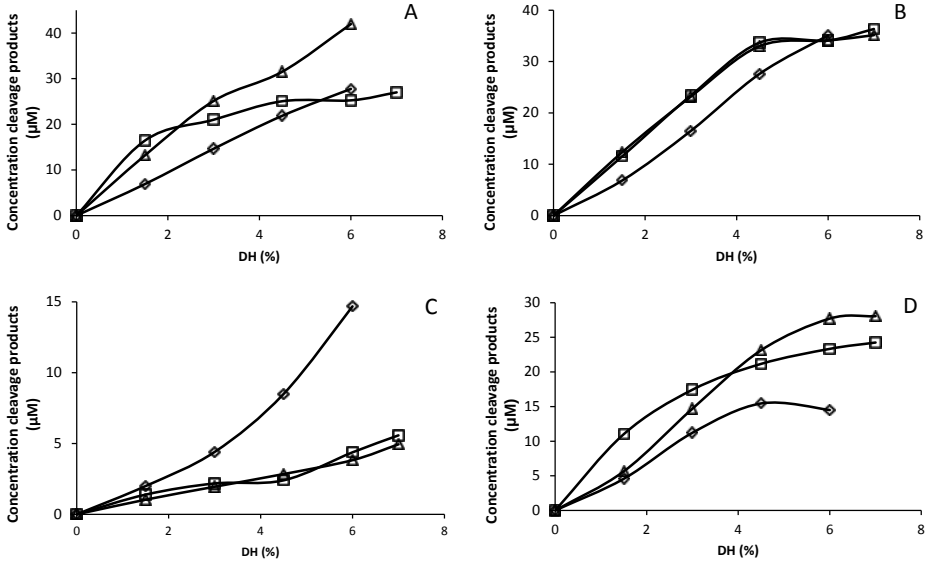


Figure 5. Concentration of cleavage products as a function of DH for β-lactoglobulin cleavage sites (A) 45; (B) 74; (C) 157 and (D) 158, for the different pH of hydrolysis, (◊) pH 7; (□) pH 8 and (△) pH 9.

As for the peptides, there are large differences in the effect of pH on the concentration of cleavage site products for different cleavage sites. The concentration of cleavage

products of cleavage site 74 is the same for all pH of hydrolysis as a function of the DH. (**figure 5B**) The concentration of cleavage products for cleavage site 157 is higher at pH 7 than at pH 8 and 9 for all DH values. (**figure 5C**) For cleavage site 158, a lower concentration of the cleavage products is obtained at pH 7 than at pH 8 and 9. (**figure 5D**) The apparent cleavage rate $k_{i,app}$ and the rate of selective hydrolysis k_i are determined from the concentration of cleavage products as a function of the time of hydrolysis. (equation 7, **figure 6**)

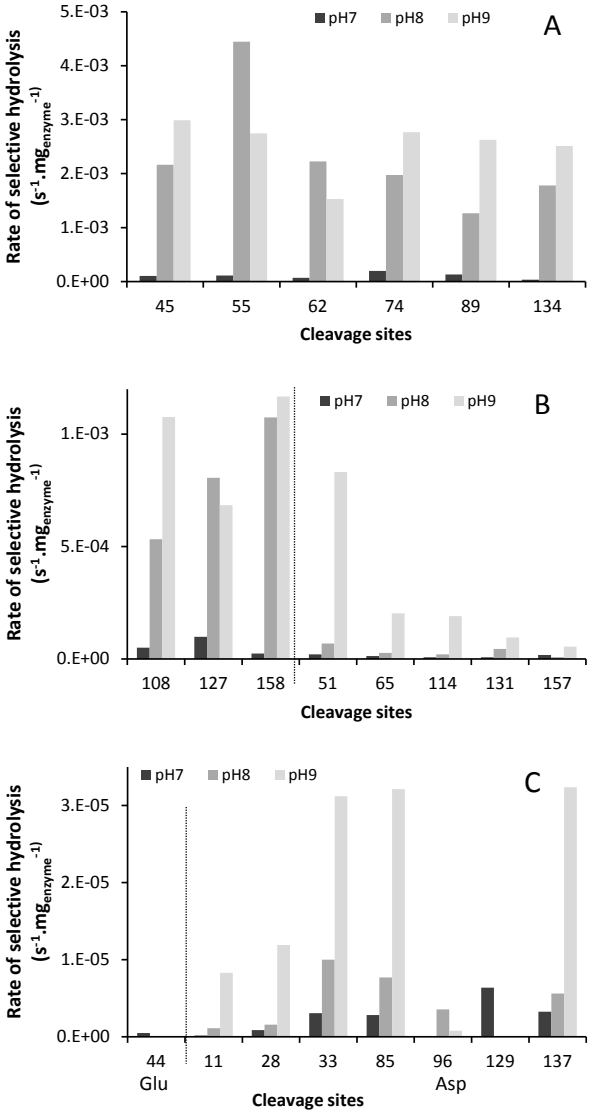


Figure 6. Rate of selective hydrolysis k_i of the different cleavage sites for the different pH of hydrolysis of 1 % WPI by BLP.

The cleavage sites are divided into five groups based on a high or low rate of selective hydrolysis. The first two groups have an average rate of selective hydrolysis of $1.6 \cdot 10^{-3}$ $s^{-1} \cdot mg_{enzyme}^{-1}$ (**figure 6A**) and of $3.0 \cdot 10^{-4}$ $s^{-1} \cdot mg_{enzyme}^{-1}$. (**figure 6B**) This second group has 2 subclasses with average rates of $6.1 \cdot 10^{-4}$ $s^{-1} \cdot mg_{enzyme}^{-1}$ and $1.1 \cdot 10^{-4}$ $s^{-1} \cdot mg_{enzyme}^{-1}$.The third group with the lowest average rate of selective hydrolysis ($4.9 \cdot 10^{-5}$ $s^{-1} \cdot mg_{enzyme}^{-1}$) corresponds to aspartic acid residues and one glutamic acid residue. (**figure 6C**) Finally a fifth group is formed by cleavage sites Glu-112 and 4 cleavage sites next to aspartic acid residues: 53, β-lg(A)64, 98 and 130 for which no cleavages were found. Based on the enzyme specificity, cleavages are expected on these cleavages sites. For the first four groups an increased rate of selective hydrolysis is found with increasing pH. (**figure 6**) This is expected based on the overall rate of hydrolysis determined based on the DH curves for the different pH of hydrolysis. (**figure 1**) For the cleavage sites after glutamic acid residues, the average rate is $5.9 \cdot 10^{-5}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ at pH 7; $1.2 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ at pH 8; and $1.4 \cdot 10^{-3}$ $s^{-1} \cdot mg^{-1}_{enzyme}$ at pH 9.

Determination of the selectivity

Using the rate of selective hydrolysis, the enzyme selectivity towards the cleavage sites was calculated (equation 9). For all condition of hydrolysis, it is clear that there are large differences in the selectivity towards the different cleavage sites. The enzyme selectivity varies from 15 % to 0.003 % and to 0 for some cleavage sites. (**table 2**)

Table 2. Effect of pH on the relative selectivity (%) of the cleavage sites

	cleavage sites	pH		
		7	8	9
increase	**45**	11.53	13.14	15.25
up and stable	**134**	3.68	10.81	12.83
	158	2.67	6.52	5.96
up and down	**55**	12.56	27.00	14.02
	62	7.71	13.51	7.80
	96	n.d.	0.022	0.004
down and stable	**74**	21.97	11.99	14.13
	127	11.02	4.90	3.49
	131	0.783	0.269	0.490
	157	1.98	0.050	0.281
	129	0.474	n.d.	n.d.
	44	0.055	n.d.	n.d.
down and up (Glu)	**89**	14.78	7.69	13.41
	108	5.62	3.23	5.49
	51	2.25	0.418	4.24
	65	1.40	0.161	1.03
	114	0.783	0.125	0.972
down and up (Asp)	**11**	0.011	0.007	0.042
	28	0.064	0.010	0.061
	33	0.227	0.061	0.159
	85	0.210	0.047	0.164
	137	0.241	0.034	0.165

n.d. peptides were analyzed until a DH of 7 %, for certain cleavage sites no corresponding peptides were found, indicating that the selectivity is < 0.003 %.

The cleavage sites are divided into 5 groups based on the behavior of the enzyme selectivity as a function of the pH. (**table 2**) The selectivity of the enzyme towards cleavage sites 45 is increasing with increasing pH. (**figure 7A, table 2)** Furthermore, 10 out of 22 cleavage sites show a clear minimum with low selectivity at pH 8 (**figures 7E and 7F**) and high selectivity at pH 7 and 9. For 3 cleavage sites, a clear optimum is observed at pH 8, and low selectivity at pH 7 and 9 (e.g. cleavage site 55). (**figure 7C**) To try to understand the different types of behavior and the changes in selectivity, the primary structure of the substrate was considered.

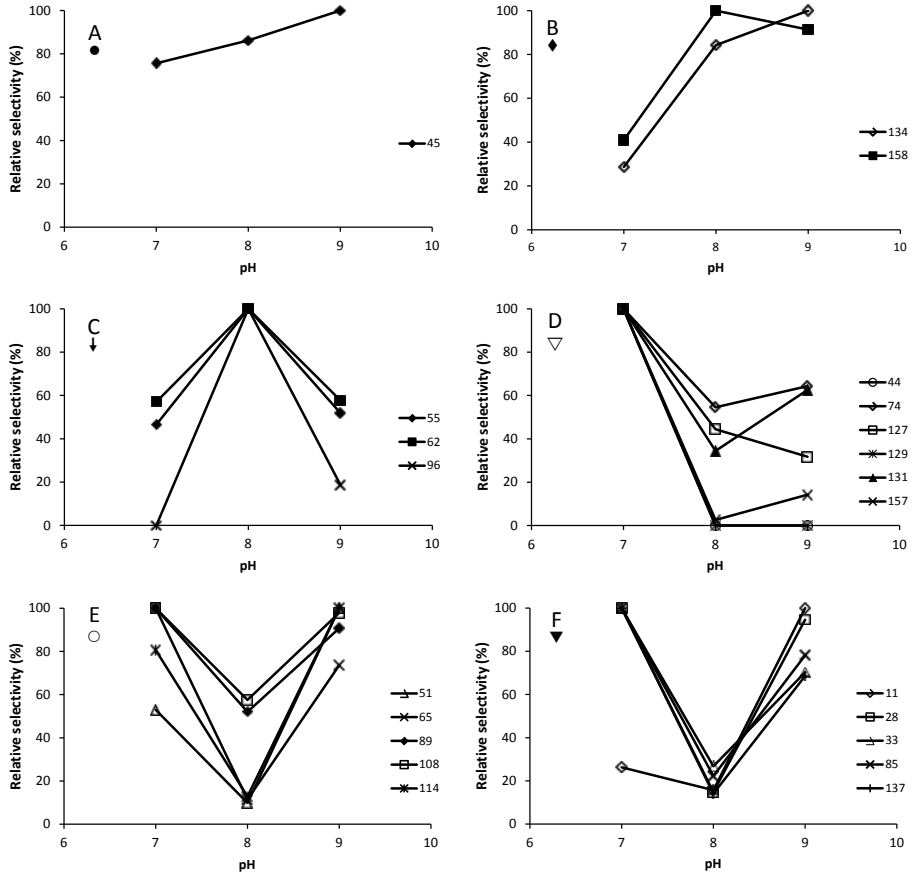


Figure 7. Relative selectivity as a function of pH for the different cleavage sites in β-lactoglobulin for hydrolysis of 1 % WPI by BLP at three pH values.

On three parts of the β-lactoglobulin sequence, a combination of two sequential cleavage sites is found (44E-45E; 129D-130D and 157E-158E). At pH 8 and 9 the first cleavage site of each sequence (i.e. 44E, 129D and 157E) is not cleaved or cleaved with a lower selectivity than at pH 7. (**figure 7D, table 2)** This seems to be due to the fact that the BLP enzyme is hindered by the presence of negatively charged amino acid

residues at the P1' position. (**figure 8**) This has been previously suggested based on the rate of hydrolysis of synthetic peptides [7]. However, in the pH range 7-9, the charge states of Glu and Asp is not expected to change. The reason for this observation may then be sought in the active site of the enzyme. The active site of BLP contains the three residues Ser, His and Asp [16]. The pKa of the side chain of the histidine residue is 6.0. Consequently, there is still a significant proportion (9 %) of His with a positive charge at pH 7 while there are 10 and 100 times less charged His side chains at pH 8 and pH 9, respectively. This decreased protonation of His may affect the interaction with the negatively charged substrate amino acids (Glu, Asp). While the changes in the charge state of His in the active site of the enzyme is the same for all peptides, it probably has an influence in the presence of a negative charge on the substrate. In this way, the negative charge on position P1' might be less unfavored at pH 7 than at pH 8 and pH 9. Consequently, the cleavage sites (44, 129 and 157) with a negative residue on P1' have a higher selectivity at pH 7 than at pH 8 and 9.

Figure 8. Amino acid sequence of β-lactoglobulin. The symbols refer to the different type of behavior of the selectivity as a function of the pH of hydrolysis in figure 7.

For the other cleavage sites, by looking at 3 amino acid residues on both side of the cleavage site, there seems to be no correlation between primary sequence, and trend of the selectivity as a function of pH. (**figure 8**) Moreover, the diversity of selectivity behavior suggests that the charge state of the subsite is not the main influence on the selectivity.

Some of the changes in selectivity as a function of pH observed in this study can be explained by changes of the charge state in the active site of the enzyme. This is in contrast with reported data on hydrolysis of casein by trypsin at different pH [5]. In that study the charge of the amino acid (Arg and Lys) next to the cleavage site was affected by the changes in the pH (7-10) of hydrolysis [5]. Different behavior in the formation of peptides was also observed with optimum pH for the formation of some of the peptides. Finally, it should also be noted that the mechanism is different from pH 7 to 9 with respect to the intact protein. At pH 7, the intact protein is less favored and the intermediate peptides are not accumulated. In this type of mechanism, which can be correlated to the one-by-one mechanism, all putative cleavages sites are used at an earlier DH than in the zipper mechanism. This might also partly explain why 44 and 129 are annotated at pH 7 and not at pH 8 and 9.

Conclusions

By changing the pH of the hydrolysis, not only the rate of hydrolysis, but also the mechanism of hydrolysis is changed. The rate of hydrolysis of intact protein as a function of DH was found to decrease at pH closer to the iso-electric point, where the protein stability is the highest. The selectivity towards the different cleavage sites varied from 15 % to 0.004 % or 0 for some cleavages sites. With increasing pH, the selectivity towards certain cleavage sites increases, while for others it decreases. The changes in selectivity as a function of pH were significant (up to 80 % increase or decrease). These changes are, however, difficult to predict. The influence of pH on the mechanism of hydrolysis should therefore be considered when studying the optimum pH of hydrolysis.

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Chapter 7

Spontaneous, non-enzymatic breakdown of peptides during enzymatic protein hydrolysis

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Abstract

It is expected that during the hydrolysis of proteins with specific enzymes only peptides are formed that result from hydrolysis of the specific cleavage sites. In reality, it is quite common to find a-specific peptides. These are formed after cleavage of peptide bonds after amino acids that are not part of the specificity of the enzyme. Such observations are often ignored, or explained by the impurity of the enzyme preparation. However, in recent work even with the quite specific (for Glu and Asp) *Bacillus licheniformis* protease (BLP), 13 peptides out of 77 identified were found to be a-specific peptides. These were formed after degradation of 6 specific peptides, after 5 different types of amino acids (Gln, Ala, Lys, Met, Ser). The fact that other peptides were not affected suggested that the cleavages were not the result of a contamination with a different enzyme. It has been described that certain peptide sequences in a small number of (metabolic) peptides may have a certain instability that results in autolytic degradation, or spontaneous cleavage. The observations in the β-lactoglobulin hydrolysate indicated that even peptides resulting from the hydrolysis of commonly used proteins such as β-lactoglobulin may be susceptible to such spontaneous cleavage. To test if the spontaneous cleavage had a role in the formation of a-specific peptides during the hydrolysis of β-lactoglobulin, the parental peptides were synthesized and incubated in the absence or presence of BLP. Surprisingly, 5 out of the 4 synthesized peptides were indeed spontaneously cleaved under the mild conditions used in this study (i.e. 40 °C and pH 8). This shows that peptides are less stable than typically considered. The rate of cleavage on the a-specific bonds was found to be enhanced in the presence of BLP. This suggests that the formation of a-specific peptides is not due to side activity but rather an enhancement of the intrinsic instability of the peptides.

Introduction

Specific enzymes hydrolyze peptide bonds after 1 or 2 amino acids. The best known example of a specific protease is trypsin, since it is considered to be specific for Lys and Arg. Nevertheless, in hydrolysates obtained from such specific proteases, a number of a-specific peptides have been annotated. For instance, during hydrolysis by trypsin cleavages after Tyr, Trp and Phe are identified e.g. at pH 7.5 [1] or pH 8 [2]. Even in proteomics studies in which sequence grade trypsin was used, such specific cleavages have been annotated [3]. A-specific cleavage products were also identified in hydrolysates from BLP (*Bacillus licheniformis* protease), which is specific for Glu and Asp residues [4]. The a-specific cleavages were found to occur after four different amino acids in β-casein: 52-Phe; 128-Thr; 188-Gln; and 192-Leu [5]. For both enzyme preparations, the a-specificity was attributed to residual activities present in the enzyme preparation. For trypsin hydrolysis, the residual side activity was assumed to be due to chymotrypsin, which is specific for large hydrophobic amino acids (Trp, Tyr, Phe). For BLP hydrolysis, the a-specific cleavages were attributed to the remaining Subtilisin, which has a preference for aromatic residues (Phe, Tyr and Trp) and Leu residues [6]. Even using a purified BLP preparation, a-specific peptides were found in hydrolysates of β-lactoglobulin by BLP (chapter 5). Surprisingly, the cleavages of a-specific cleavage bonds are only occurring on a limited number of cleavage sites (chapter 5). If the cleavages were the results of hydrolysis by another enzyme present, more of these cleavages would be obtained. In the latter study the molar concentration of all peptides was followed as a function of DH. Based on the concentration of the peptides, it was shown that the a-specific cleavages occur for a limited number of peptides after different types of amino acids. The cleavages occurred at positions 59 (Gln-Lys) in peptide β-lg[56-62]; 80 (Ala-Val) in peptide β-lg[75-89]; 141 (Lys-Ala); 145 (Met-His) and 150 (Ser-Phe) in peptides β-lg[135-157], β-lg[138-157], β-lg[135-158] and β-lg[138-158]. Interestingly, in longer peptides containing the same peptide bond (i.e. [63-89] or [113-157]), the peptide bonds were not cleaved. Moreover, the same amino acids in other parts of the sequence (e.g. 138 (Lys-Ala)) were not used as cleavage site. Based on this, it is unlikely that the cleavages are the result of side activity of the enzyme preparation or impurities. Rather, it is considered to be the result of a type of autolytic mechanism that only occurs in certain specific peptide sequences. Still, in enzymatic protein hydrolysates, it is difficult to clearly identify the formation of these a-specific peptides, since they are in a complex mixture of peptides.

Such so-called spontaneous cleavages have been observed in specific isolated cases, such as the vasoactive intestinal peptide, a peptide hormone consisting of 28 amino acid residues [7]. This peptide showed an intrinsic autolytic activity and was cleaved at a rate of $1.5 \cdot 10^{-5}$ s^{-1} (at neutral pH and 38 °C). The spontaneous cleavage was inhibited when the secondary structure of the peptide was changed by addition of sodium dodecyl sulfate. Another well-known example is the group of proteins referred to as inteins. Inteins are a part of the sequence of specific proteins involved in protein or RNA splicing [8]. The inteins are released from the rest of the protein by two protein

cleavage events, as a result of autocatalytic cleavage [9]. These examples show the spontaneous cleavage of peptides due to specific structural properties. Furthermore, slow cleavages of peptide bond Phe-Gly in peptide Phe-Phe-Phe-Gly have been observed in neutral water solutions at room temperature at a rate of $3 \cdot 10^{-9}$ s^{-1} (corresponding to a half-life of 7 years). These cleavages were shown to be the result of uncatalyzed attack of the water and not due to impurities [10,11].

Another class of spontaneous cleavage is attributed to the presence of specific amino acids (asparagine, or serine) in a peptide sequence. The bonds after Asn can be hydrolyzed after an intramolecular nucleophilic attack, following the deprotonation of the amide group of the side chain of the aspartic acid [12]. The rate of this reaction is $1.6.10^{-7}$ s^{-1} at pH 8 in MES buffer and 37 °C. Furthermore, the cleavage of peptide bonds on the N-terminal side of serine residues are considered to be the result of an intramolecular attack involving the hydroxyl group of the serine residue [13,14]. This reaction occurs at 37 °C and neutral pH at a rate of $1.7 \cdot 10^{-7}$ $\mu mol \cdot s^{-1}$ (equivalent to 25 % conversion after 500 hours) and is 10 times faster at 60 °C.

From the above it becomes clear that not all peptides are intrinsically stable. On the contrary, several peptides are known to undergo spontaneous cleavage. However, the phenomenon is not typically considered to occur during enzymatic hydrolysis of proteins.

Five peptides were synthesized to further study the a-specific cleavages of certain peptides bonds observed previously in hydrolysates of β-lactoglobulin by BLP (chapters 5 and 6). Firstly, the formation and kinetics of a-specific cleavage in the presence of the enzyme was determined to confirm the observations from the complex mixture (chapters 5 and 6). In addition, the synthesized peptides were also incubated individually in the absence of enzyme, to test if there was indeed an intrinsic instability of the peptides.

Materials and Methods

Materials. BLP (*Bacillus licheniformis* protease - NS-37005) was obtained from Novozymes (Bagsvaerd, Denmark). The BLP (4.5 % (w/w) protein, by Dumas method Nx6.25; activity of 0.3 AU/mg/min as determined by azocasein assay [15]) was partly insoluble and was fractionated as described before (chapter 3). The freeze dried, water soluble material was found to contain 60 % (w/w) protein (Nx6.25). Based on the UV_{214} area from RP-UPLC-MS, it was found that 78 % of the total UV area corresponds to the enzyme BLP (23.6 kDa) and 14 % to the pro-peptide (6.9 kDa) as described previously (chapter 3). The enzyme has an activity of 3.9 AU/mg/min as determined by the azocasein assay. Peptides β-lg[135-157] and β-lg[138-157] were synthesized by Biomatik (Wilmington, DE, USA) and stored at -20 °C. (**table 1**) Three other peptides (described below) were synthesized in-house, using Fmoc-protected amino acids and Fmoc-Glu(OtBu) Wang resin (0,51 mmol/g loading), which were purchased from Novabiochem. HBTU, DIEA, Fmoc-Asp(OtBu) Wang resin (0.4-0.9 mmol/g loading),

piperidine, TFA, triisopropylsilane (TIS), β-lactoglobulin (purity > 90 %) and all other chemicals were of analytical grade and purchased from Sigma.

Table 1. Sequence and purity of the synthesized peptides.

peptide	sequence	calculated mass	observed m/z		purity
			$[M+H]^+$	$[M+2H]^{2+}$	
β-lg[56-62]	ILLQKWE	928.5	929.8	465.4	70%
β-lg[75-89]	KTKIPAVFKIDALNE	1686.0	1687.5	844.2	70%
β-lg[75-85]	KTKIPAVFKID	1258.8	1259.9	630.7	85%
β-lg[135-157]	KFDKALKALPMHIRLSFNPTQLE	2696.5		1349.2	96%
β-lg[138-157]	KALKALPMHIRLSFNPTQLE	2306.3		1154.1	90%

Synthesis of the peptides

Peptides β-lg[56-62], β-lg[75-89] and β-lg[75-85] were synthesized at Parma University, Italy, using Fmoc-SPPS on Wang resins preloaded with the C-terminal amino acids. The syntheses were carried out using an automated peptide synthesizer (Syro I, Biotage, Uppsala, Sweden). Amino acid coupling was performed in presence of 5 equiv. of amino acid, 10 equiv. of DIEA and 4.7 equiv. of HBTU to the initial loading of the resin. Fmoc-deprotection was achieved by treatment of the resin with 40 % (w/v) piperidine. After completion of peptide syntheses, the peptide-resins were dried under vacuum; cleavage of the peptides from the resins was achieved by treatment with a mixture of TFA, TIS and water (95: 2.5: 2.5) for 2 h at room temperature. The resins were removed by filtration and washed with TFA. The combined filtrates were then dried under N_2 flux. Cold ethyl ether (5 °C) was added to the residues to precipitate the unprotected peptides.

The products obtained were characterized by RP-UPLC-ESI-MS. The purity was determined based on the area of the peptide in the UV_{214} compared to the total UV area. (**table 1**) No sign of spontaneous degradation was observed during the synthesis of the compounds.

Incubation of the synthetic peptides

Solutions of peptide β-lg[56-62] (350 μM), peptide β-lg[75-85] (350 μM), peptide β-lg[75-89] (350 μM), peptide β-lg[135-157] (200 μM) and peptide β-lg[138-157] (200 μM), were prepared in 7 mL millipore water. All peptides were incubated separately in the absence or presence of 20 μL BLP (diluted freshly at 5 % (w/v)) in a pH-stat at pH 8.0 and 40 °C using 0.1 M NaOH to adjust the initial pH and to keep the pH constant during the incubation. As control, intact β-lactoglobulin (1 % (w/v) or 540 μM) was incubated alone under the same conditions. Samples were taken at different time points during incubation (0, 30, 90, and 180 min). For β-lg[135-157] and β-lg[138-157] incubated in the absence of enzyme an additional sample was taken after 18 hours. Samples without enzyme were directly cooled to -20 °C and samples containing enzymes were inactivated at pH 2 by addition of 3 M HCl and the pH was adjusted back to 8.0 after 10 minutes of inactivation. All samples were stored at -20 °C before analysis by UPLC-MS.

To test the effect of the pH inactivation on the stability of the samples, the pH of one of the samples obtained from incubation of β-lg[56-62] in the absence of enzyme was also adjusted to pH 2 and back to pH 8 before injection on RP-UPLC-MS. No effect of the pH was found on the products formed. To test if the mechanism of cleavage is due to nucleophilic attack, peptide β-lg[56-62] was incubated alone at 40 °C and pH 9. The same peptide was also incubated in the presence of CH_3NH_2 with a molar ratio CH_3NH_2/peptide of 20 at pH 8 and 40 °C.

RP-UHPLC-ESI-QTOF-MS

Samples were analyzed using a Waters Acquity UPLC System (Waters Corporation, Milford, MA, USA). Eluent A was 1 % (v/v) acetonitrile (ACN) containing 0.1 % (v/v) trifluoroacetic acid (TFA) in Millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. The samples were analyzed on a Waters Acquity UPLC BEH 300 C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 x 50 mm, 1.7 µm particle size, Waters). The samples obtained during peptide incubation were diluted two times in eluent A (to a final concentration of 100-175 µM). The samples obtained during incubation of β-lactoglobulin were diluted to a final concentration of 0.1 % (w/v) in eluent A. All samples were centrifuged (10 min, 19000 g, 20 °C) before injection (4 µL). The elution profile was as follows: 0-2 min isocratic on 3 % B; 2-10 min linear gradient from 3-22 % B; 10-16 min linear gradient 22-30 % B; 16-19 min linear gradient 30-100 % B; 19-24 min isocratic on 100 % B; 24-26 min linear gradient 100-3 % B and 26-30 min isocratic on 3 % B. The flow rate was 350 µL·min^{-1}. Detection was done using a PDA, which was constantly scanning the range 200-400 nm at a 1.2 nm resolution with 20 spectra per second.

Samples from UPLC were directed to an online Synapt high definition mass spectrometer (Waters Corporation) equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). The settings were the same as described before (chapter 4). MS and MS/MS (MSe method) were performed in positive mode between m/z 100-2000 with a scan time of 0.3 seconds. UV and MS data were acquired using MassLynx software v4.1 (Waters).

Peptide Quantification

The peptides were quantified based on the UV signal at 214 nm, using equation (1)

(1) $$C_{peptide} = 1 * 10^6 \left(\frac{A_{214}}{\varepsilon_{214} l V_{inj} k_{cell}}\right) Q$$

In which $C_{peptide}$ (µM) is the concentration of peptide, A_{214} (AU.min) is the UV peak area at 214 nm, V_{inj} (µL) is the volume of sample injected, Q the flow rate in µL·min^{-1}, l is the path length of the UV cell which is 1 cm according to the manufacturer. k_{cell} = 0.66 is a constant depending on the geometry of the UV detector used. The value of k_{cell} was previously determined using pure peptides [16]. ε_{214} is the molar extinction coefficient at 214 nm of the peptide calculated as described before [17]. The concentration was corrected for evaporation during incubation (100 µL.h^{-1}).

Results and Discussion

Detection of a-specific peptides in a WPI hydrolysate by BLP

The hydrolysates obtained at different DH values during hydrolysis of 1 and 5 % (w/v) whey protein isolate (WPI) by BLP were analyzed in detail. Over all hydrolysates, 77 peptides resulting from the hydrolysis of β-lactoglobulin were annotated and quantified (chapter 5). Among the 77 annotated peptides, 13 a-specific peptides were detected. With increasing substrate concentration and with increasing DH, the concentration of a-specific peptides increased. (**table 2**) It seems that the concentration of a-specific peptides is more dependent on time than on the DH or the initial substrate concentration. The a-specific peptides are not present at the beginning of the hydrolysis. This suggests that the peptides are formed from intermediate peptides.

Table 2. Concentration (μM) of a-specific peptides and parental peptides from β-lactoglobulin annotated during hydrolysis of 1 % and 5 % (w/v) WPI with BLP as a function of the degree of hydrolysis (DH) (chapter 5). *indicates the specific peptides.

Sequence in β-lactoglobulin	Observed mass (Da)	Theoretical mass (Da)	1 % WPI					5 % WPI				
			Degree of hydrolysis (%)									
			1.5	3	4.5	6	7	1.5	3	4.5	6	7
Stable peptides												
[46-51]*	683.397	683.385	1.17	3.44	6.96	23.2	30.6	1.90	4.96	18.5	32.8	27.6
[52-55]*	432.189	432.186		4.00	6.58	16.6	24.2	3.91	4.89	11.7	29.8	28.5
[66-74]*	1002.560	1002.553		3.43	13.1	24.8	28.4	1.31	8.17	23.6	31.4	27.1
Unstable peptides												
[56-62]*	928.537	928.538	15.6	27.0	32.9	26.2	14.9	14.9	20.4	20.6	8.70	
[56-59]	485.339	485.321		2.22	3.22	8.46	14.8		2.18	4.61	12.20	13.69
[60-62]	461.231	461.227		0.46	1.18	7.08	11.7		1.93	1.93	13.12	19.48
[75-85]*	1258.772	1258.765				0.99	5.82			0.86	8.39	
[75-89]*	1685.974	1685.972	5.86	15.0	30.2	23.7	12.5	6.66	17.0	27.0	18.4	0.185
[75-80]	656.435	656.422			1.35	9.24	21.6		0.62	2.05	12.2	30.5
[81-89]	1047.582	1047.560				5.34	3.82			0.82	3.98	5.73
[135-157]*	2696.484	2696.502	0.79	0.56	0.78	4.77	2.48		0.47	0.48	4.28	0.15
[135-158]*	2825.526	2825.535	6.99	14.1	21.2	7.07	1.88	6.99	13.6	16.5	10.2	0.57
[138-157]*	2306.293	2306.308				0.45	0.72				1.38	2.38
[138-158]*	2435.336	2435.316				1.24	0.65				2.25	0.91
[135-141]	848.519	848.512					1.65					
[138-145]	870.544	870.536									1.18	10.3
[142-157]	1865.990	1865.982					1.04					
[142-158]	1995.010	1995.025				1.29	2.09					
[146-150]	624.383	624.371										5.92
[146-157]	1453.774	1453.768					3.82					3.58
[146-158]	1582.822	1582.810				2.53	4.96				1.35	2.49
[151-157]	847.419	847.408										4.99
[151-158]	976.460	976.450										1.10

For all a-specific peptides, the specific peptides from which they were formed were identified (chapter 5). This was based on the fact that the concentration of the parental peptides from which the a-specific peptides are derived, β-lg[56-62], β-lg[75-85], β-lg[75-89], and β-lg[138-157] is significantly decreased in the later stages of hydrolysis. Since these parental peptides do not contain Asp or Glu residues, the decrease in their concentration was not expected. For other peptides that do not contain Asp or Glu

residues, such as β-lg[46-51], β-lg[52-55], or β-lg[66-74] the concentration only increases with time and no products resulting from their cleavages were found in the hydrolysates. (**table 2**) This confirms that the decrease in concentration of peptides β-lg[56-62], β-lg[75-85], β-lg[75-89] and β-lg[138-157] is indeed due to the formation of a-specific peptides. Moreover, it confirms that not all peptides formed during the hydrolysis are further cleaved on a-specific cleavage sites. An additional observation is that the peptide bonds 59 (Q-K); 80 (A-V); 141 (K-A); 145 (M-H) and 150 (S-F) seem to be stable in other peptides (and in the intact β-lactoglobulin). This shows that the a-specific cleavage does not only depend on the peptide bond, but also on the peptide in which it is present. This observation is further supported by the fact that the amino acids after which spontaneous cleavage are observed (Q, A, K, M, S) are not observed as cleavage sites in other parts of the protein sequence. For instance, peptide bond 141 (K-A) was found to be cleaved, but no cleavages were annotated for the exact same bond at position 138 (K-A). In addition it indicates that this type of cleavage is in fact not a-specific. Therefore, it is not expected that the cleavages are the result of enzymatic side activity in the enzyme preparation. To confirm the observations obtained in the hydrolysates, the parental peptides were synthesized. (**table 1**)

Incubation of the synthesized peptides in presence of BLP

Each synthesized peptide was incubated in the presence of BLP. The BLP used is quite pure (78 %). It also contains 14 % of pro-peptide (6.9 kDa, accession number P80057 - www.uniprot.org), which is considered inactive. From the quantification of peptides at different time points, it is observed that β-lg[75-89] is first cleaved by the enzyme next to the aspartic acid, position 85 (D-A), for which the enzyme is specific. (**figure 1A**)

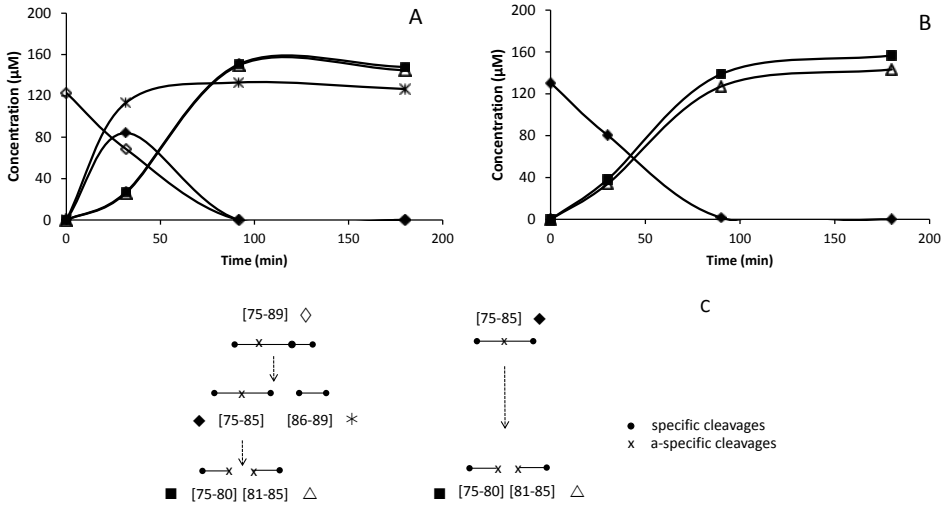

Figure 1. Quantification of the peptides obtained in the presence of the enzyme BLP during incubation of (A) β-lg[75-89] (◊) and (B) β-lg[75-85] (♦); derived peptides are: (■) β-lg[75-80]; (Δ) β-lg[81-85]; (✳) β-lg[86-89]. (C) Scheme of the pathways of cleavages.

This leads to the formation of β-lg[75-85] and β-lg[86-89]. The peptide β-lg[75-85] formed is further cleaved at an a-specific cleavage site resulting in the formation of β-lg[75-80] and β-lg[81-85]. During incubation of the peptide β-lg[75-85], in presence of BLP, the two same a-specific peptides β-lg[75-80] and β-lg[81-85] are obtained (**figures 1B**). In both cases, the parental peptides are rapidly hydrolyzed in the first 90 minutes of incubation. After three hours of incubation, no parental peptide was left. The a-specific cleavage on position 80 (A-V) is the same cleavage as was previously observed in the hydrolysates of β-lactoglobulin. (**table 2**)

For the peptide β-lg[138-157], a more complex breakdown pattern than for β-lg[75-89] was observed when incubated with BLP. (**figure 2**) The peptide was first completely cleaved on position 145 (M-H) leading to the formation of peptides β-lg[138-145] and β-lg[146-157] in the first 30 minutes of the incubation. Both peptides were then subsequently cleaved; peptide β-lg[138-145] was cleaved on 141 (K-A) and peptide β-lg[146-157] was cleaved at position 150 (S-F). (**figure 2B**) The two intermediate peptides, β-lg[138-145] and β-lg[146-157] are accumulated in the first part of the incubation (from 0 to 30 min). After three hours of incubation, these peptides were completely converted into 4 a-specific peptides.

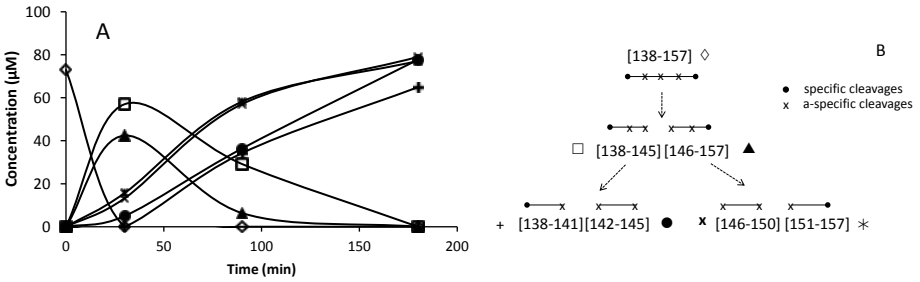

Figure 2. (A) Concentration of the peptides obtained during incubation of β-lg[138-157] in presence of the enzyme BLP. (◊) β-lg [138-157]; (□) β-lg [138-145]; (▲) β-lg [146-157]; (x) β-lg [146-150]; (∗) β-lg [151-157]; (+) β-lg [138-141]; (•) β-lg [142-145] and (B) pathway of cleavages of β-lg[138-157] in presence of the enzyme BLP.

The peptide β-lg[135-157], even showed three parallel cleavage pathways during its incubation with BLP. (**figure 3**) The peptide is cleaved after the aspartic acid on position 137 (D-K) as expected by the enzyme specificity. The resulting peptide β-lg[138-157] is then completely degraded as described above.
After 3 hours of incubation both specific peptides β-lg[135-157] and β-lg[138-157] are completely degraded. At the same time as the specific cleavage occurred, a-specific cleavages occurred on the parental peptide β-lg[135-157] on bond 141 (K-A), and 145 (M-H), resulting in two alternative cleavage pathways. The peptide β-lg[146-157] was further cleaved to form β-lg[146-150] and β-lg[151-157]. (**figure 3**)

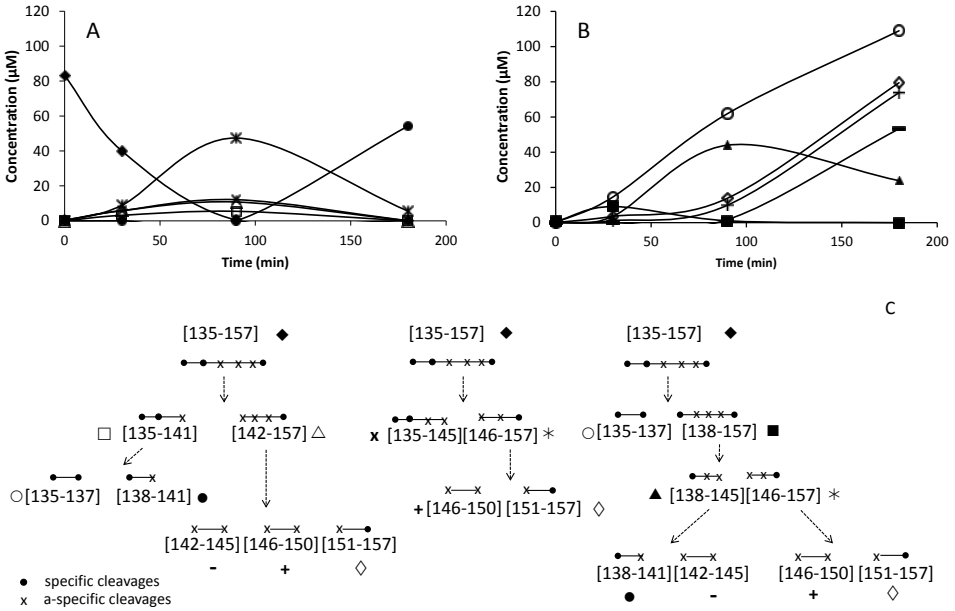

Figure 3. Quantification of the fragments obtained during incubation of β-lg[135-157] in presence of the enzyme BLP for peptide. (A) (♦) β-lg [135-157]; (□) β-lg [135-141]; (Δ) β-lg [138-145]; (+) β-lg[146-150]; (◊) β-lg[151-157], (○) β-lg[135-137] and (-) β-lg[142-145]. (C) Scheme of the 3 parallel pathways of cleavages.

Peptides β-lg[135-141] and β-lg[142-157] are also further cleaved rapidly as seen by the low accumulation of these peptides. The cleavages on the bonds 141 (K-A), 145 (M-H) and 150 (S-F) observed during incubation of β-lg[138-157] and β-lg[135-157] correspond to the annotated cleaved bond in the β-lactoglobulin hydrolysate. (**table 2**) In addition, it is important to note that for peptide β-lg[135-157], a-specific cleavages are observed in parallel to the specific cleavage.

Peptide β-lg[56-62] was cleaved at position 59 (Q-K). (**figure 4**) The peptide is degraded rapidly in the first 30 minutes of the incubation and is completely degraded after 3 hours of incubation. This indicates a complete degradation of the parental peptide into two peptides: β-lg[56-59] and β-lg[60-62].

Summarizing, for the five synthesized peptides the same a-specific cleavages are found as in the original β-lactoglobulin hydrolysate. This confirms that the identified parental peptides were indeed the source of the observed a-specific peptides.
Still, since the observed cleavages do not agree with the known enzyme specificity, it is suspected that these peptides have an intrinsic instability. To test if the peptides are stable, the synthesized parental peptides were also incubated at the same conditions in the absence of enzyme.

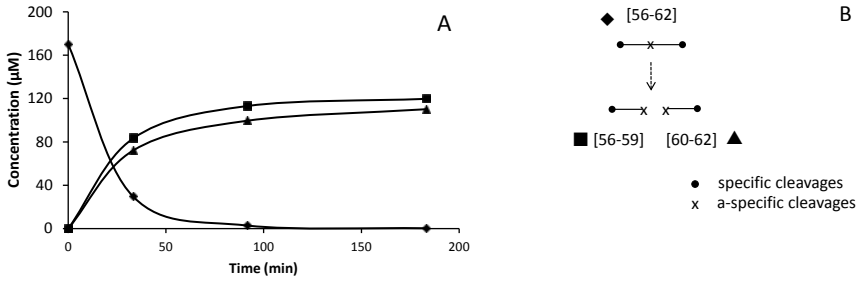

Figure 4. Quantification of the fragments obtained during incubation of the peptide β-lg[56-62] with BLP (♦) β-lg[56-62] (■) β-lg[56-59] and (▲) β-lg[60-62].

Incubation of the synthesized peptides alone

The peptide β-lg[75-89] was stable during 3 hours of incubation when incubated alone. In contrast, the peptide β-lg[75-85], was partly (0.3 %) converted into peptides β-lg[75-80] and β-lg[81-85] after 3 hours of incubation. This shows that the peptide bond 80(A-V) is stable when present in the peptide β-lg[75-89] as well as in the intact β-lactoglobulin, but is slowly spontaneously cleaved in β-lg[75-85]. (**figure 5**)

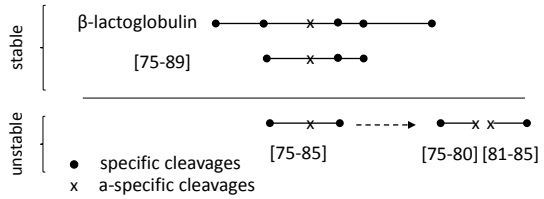

Figure 5. Scheme of the stability and instability observed on peptides β-lg[75-89] and β-lg[75-85], when incubated individually in the absence of BLP.

Similar to β-lg[75-85], the peptide β-lg[135-157] was converted for 0.3 % after 3 hours of incubation, resulting in the formation of β-lg[135-145] and β-lg[146-157]. This confirms the spontaneous cleavage of the bond 145 (M-H). After an extended incubation of 18 hours, the concentration of the two fragments further increased with a factor 4 (total of 1.4 % conversion). In addition, traces of peptides β-lg[135-141], β-lg[142-157], β-lg[146-150] and β-lg[151-157] are found after 18 hours of incubation, resulting from cleavages on bonds 141 (K-A) and 150 (S-F). Cleavages on these bonds correspond to the cleavages observed during incubation of the peptide with BLP. (**figure 6**)

For β-lg[138-157], spontaneous cleavage on bond 145 (M-H) was also observed. The cleavage was slightly faster in this peptide than in the longer peptide β-lg[135-157]. After 3 hours of incubation, 0.6% of the parent peptide was converted into β-lg[138-145] and β-lg[146-157]. After extended incubation to 18 hours, 5 % of the parental peptide was converted. (**figure 6**)

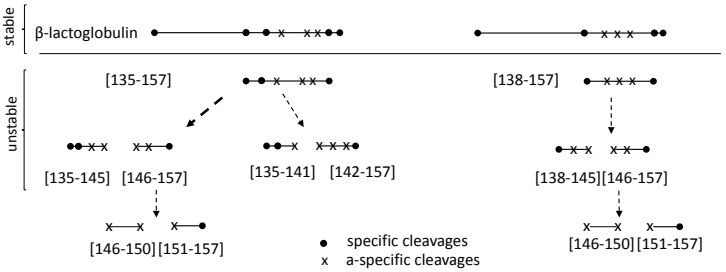

Figure 6. Scheme of the cleavages occurring on peptides β-lg[135-157] and β-lg[138-157] when incubated alone. (●) Symbolizes specific cleavages and (x) symbolizes a-specific cleavages.

For the last peptide tested, β-lg[56-62], an even higher rate of spontaneous cleavage (on bond 59 (Q-K)) was found, with 15 % conversion after 3 hours of incubation. This cleavage occurs after a glutamine. In this case, the mechanism of cleavage may be similar to described cleavages occurring on bonds next to aspartic acid and asparagine [18]. In the latter case, the cleavages are induced through the formation of a succinimide intermediate. A similar mechanism may be acting for glutamine. The cleavage after aspartic acid occurred at a rate of $1 \cdot 10^{-6}$ s^{-1} (half-life of 3.1 days) at pH 9.2 at 37 °C in MES buffer [12]. The two peptides formed (β-lg[56-59] and β-lg[60-62]) correspond as well to the peptides identified in the complete β-lactoglobulin hydrolysates. (**table 3 and figure 7**)

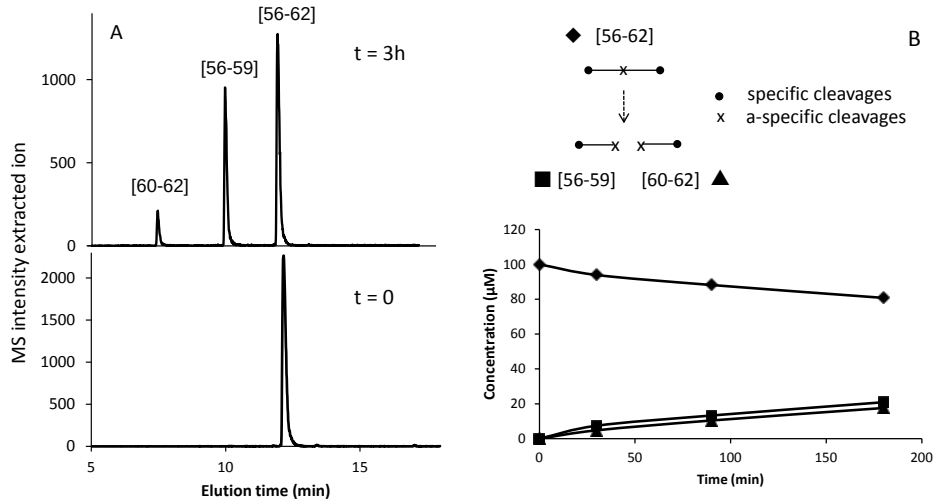

Figure 7. (A) Chromatogram of the extracted ions of the sample β-lg[56-62] at time 0 and after 3 hours of incubation alone at pH 8 and 40 °C. (B) Quantification of the fragments obtained during incubation of the peptide β-lg[56-62] (♦) β-lg[56-62] (■) β-lg[56-59] and (▲) β-lg[60-62].

In all cases, the incubation in the absence of enzyme showed the formation of the same peptides as in the presence of enzyme. Firstly, this shows that the peptides do

indeed contain an intrinsic instability, resulting in spontaneous cleavages, albeit at a slow rate. This instability depends on the peptide as seen for β-lg[75-89] and β-lg[75-85]. The very same peptide bond is stable in the first peptide but not in the second one.

Secondly, in this study a number of peptides from the β-lactoglobulin sequence were chosen, based on previous observations on the formation of such a-specific peptides during enzymatic hydrolysis. However, since 4 out of 5 of these peptides show spontaneous cleavage, it must be considered that the phenomenon is more common than typically assumed. It may even be that more of the a-specific peptides observed in other studies are the result of such spontaneous cleavage.

Mechanism of the cleavage

The spontaneous cleavages occurring here after different types of amino acid (Q, A, K, M, S) do not correspond to previously reported spontaneous cleavages (i.e. after N or S). Peptides would generally be considered to be stable under the conditions used in this study (i.e. pH 8, 40 °C, 0-18 hours). The most likely mechanism for cleavage of a peptide bond is a nucleophilic attack by water or hydroxide ion. To test if a generic nucleophile can indeed increase the rate of cleavage, β-lg[56-62] was incubated in the presence of methylamine (CH_3NH_2) at pH 8, and in a different experiment at pH 9. However, the presence of both nucleophiles did not result in a change in the rate of spontaneous cleavage (**table 3**).

Table 3. Concentration (μM) of fragments and parental peptide β-lg[56-62] incubated at different conditions (in the presence of BLP, incubated alone at pH 8; incubated alone at pH 9 and incubated alone at pH 8 in the presence of CH_3NH_2).

	ILLQKWE [56-62]				ILLQ [56-59]				KWE [60-62]			
time (min)	with enzyme	no enzyme	pH9	CH_3NH_2	with enzyme	no enzyme	pH9	CH_3NH_2	with enzyme	no enzyme	pH9	CH_3NH_2
0	100	100	75	78	0	0	0	0	0	0	0	0
30	27	93	76	80	77	7	0	7	67	5	0	2
90	3	88	74	75	103	13	7	10	91	10	1	3
180	0	80	74	75	104	21	10	13	95	17	2	5

Since the cleavages occur after different type of amino acids, it is unlikely that the mechanism of cleavage is from a chemical reaction dependent on a specific amino acid. It is rather related to the structure of the peptide, which depends on the sequence of the peptide. A tentative explanation for the cleavages is the conformation of the peptides. Due to an internal strain induced by the specific sequence, internal energy on some peptide bonds increases, leading to their slow degradation. In the case of Asn, a succinimide intermediate may be formed. This intermediate is then hydrolyzed as the result of a nucleophilic attack of water. In our case, since spontaneous cleavage is taking place, also a reaction intermediate is expected to be formed. Since the addition of a nucleophile does not accelerate the reaction it indicates that the formation of this reaction intermediate is the rate limiting step.

Conclusions

Certain peptides formed during enzymatic protein hydrolysis may be intrinsically unstable. This was shown by spontaneous cleavage of peptides occurring when incubated in the absence of the enzyme. In addition, the spontaneous cleavages observed in the absence of the enzyme are increased in the presence of the enzyme. This strongly suggests that the enzyme does actually bind the peptides at all possible locations in its active site. Consequently, cleavage occurs either if there is a specific amino acid Glu or Asp, or if the peptide bond is intrinsically unstable.

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Chapter 8

A simulation model to describe the hydrolysis of proteins by specific and a-specific proteases

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Abstract

During enzymatic hydrolysis of proteins, large numbers of different peptides are formed. To increase the understanding of the hydrolysis process, in this article, a stochastic model is presented to simulate the hydrolysis of one or more different types of proteins by one or more enzymes of which the specificity is known. The model is based on a random selection of protein (or derived peptide) molecules, followed by random selection of an amino acid on this molecule. If the chosen location fits the enzyme specificity a cleavage is performed. Simulations were performed to mimic the hydrolysis of whey protein isolate by the Glu and Asp specific protease from *Bacillus licheniformis*. The results obtained show quite a striking correspondence with the experimental result, although for certain peptides and cleavage sites differences are observed. The high correspondence seemed to be due to the fact that the chance for each cleavage site to be hydrolyzed depends on the distribution of cleavage sites over the amino acid sequence. Since it was experimentally observed that a decrease of pH (from 9 to 7) resulted in a decreased accessibility of the intact protein, additional simulations were performed where this parameter was included. When the accessibility was taken into account, an even better correlation between simulation and experiment was obtained. Still, some cleavage sites were hydrolyzed faster or slower than in the model. Such observations can be used to more clearly identify whether the experimentally observed peptide release kinetics can be attributed to (expected) random mechanism of hydrolysis, or if the selectivity of the enzyme to certain cleavage sites is lower than expected.

Introduction

Enzymatic hydrolysis is used for different purposes, such as the reduction of allergenic potential or the improvement of digestibility [1,2]. It is also used to produce certain specific peptides that exhibit bio-functional properties. In addition, it was recently shown that certain peptides from hydrolysates of β-lactoglobulin affect the protein unfolding, aggregation and gel properties [3]. In many of these applications, the (nutritional or techno-functional) properties of the hydrolysate are the result of a relatively small number of peptides, compared to the total number of peptides present in the hydrolysate. Despite the large number of publications on and applications of hydrolysates, little is known about the mechanism of hydrolysis of proteins. To increase the understanding of the kinetics of formation - and subsequent degradation - of the peptides during the course of the hydrolysis, a modeling approach can be used.
The hydrolysis kinetics of proteins has been described using the Michaelis-Menten model [4] or general kinetic models [5,6] by making use of the degree of hydrolysis (DH) as a function of time. In certain cases, data of peptide release kinetics have been fitted using either Michaelis-Menten or other kinetic models [7,8]. While these studies have been useful in quantifying the kinetics of the process, the approach cannot be used to predict the behavior of the system in other conditions (e.g. change of substrate, conditions, etc.). Typically to achieve such information and flexibility, a more mechanistic model should be used. One approach for such models is to simulate the hydrolysis process using a stochastic description of the process [9].

Stochastic models have already been successfully applied to describe the hydrolysis process of linear homopolysaccharides with same type of linkages [10], as well as models of branched homopolysaccharides with different linkage types (i.e. α-1,4 and α-1,6 linkages in starch) [11]. For such molecules, the description is comparatively straightforward, since all oligomers of the same size can be considered equal. Typically, such studies focus on a crude estimation of the change in the molecular size distribution. Furthermore, the enzyme has an equal chance to perform a scission at any site, since all monomeric units are equal. Consequently, the process can be described by relatively simple mathematical models. Such models assume stochastic processes and have already been shown to provide insights that can be used to understand and predict the breakdown of such linear polysaccharides under different conditions.

In the case of proteins the approaches used for homopolysaccharides cannot be applied that easily. The major reason is that the proteins are (linear) heteropolymers, which has the consequence that oligopeptides of the same number of amino acids are not necessarily identical. In addition, not all oligopeptides can be further degraded, since they may not have any cleavage sites unlike in the case of linear homopolymers. These considerations illustrate the need for a different approach towards the predictive modeling of protein hydrolysis. Such a model should, therefore, be based on the primary structure (amino acid sequence) of the proteins that are hydrolyzed. The second input parameter is the known, or assumed, specificity of the enzyme, that

describes after which amino acids (cleavage sites) the peptide bonds can be cleaved by the enzyme. The third input is the selectivity of the enzyme, which describes the relative rate at which individual cleavage sites are hydrolyzed. The selectivity depends, amongst others, on the accessibility of the substrate and the affinity towards the individual cleavage site which is determined by the neighboring amino acids as described previously (chapter 4). The benefit of such a model would be that it helps to understand and even improve the results of experimental analysis.

Even a simple hydrolysis of a single protein by one enzyme can lead to the formation of a large variety of peptides. For instance, the hydrolysis of β-lactoglobulin (β-lg) by *Bacillus licheniformis* protease (BLP), an enzyme with specificity towards Glu and Asp residues (in total N = 27 in β-lg A and N = 26 in β-lg B) can lead to the formation of 406 different peptides for β-lg A (calculated by (N+1)(N+2)/2) and 658 peptides for the combination of A and B variants. Consequently, a complete quantitative analysis of peptides in hydrolysates obtained at different time points during the hydrolysis generates a large amount of data. Since it is not known what the expected results are from such analysis, the data from experiments cannot easily be used to deduce mechanistic parameters of the hydrolysis process. Some studies use kinetic models to describe experimental data on the peptide release kinetics of several identified and (semi-)quantified peptides [7,8]. This approach does not lead directly to more detailed insight in the complete hydrolysis process for two reasons. Firstly, the release kinetics of each individual peptide is considered individually and is not directly linked to the kinetics of other peptides. Since of course in protein hydrolysis the release kinetics of all peptides are related, some observations may easily be misinterpreted. Secondly, the kinetic models are not mechanistic. While rate constants for the release of different peptides may be obtained, no real mechanistic understanding is developed on the differences between release kinetics of different peptides. An alternative to such retrospective analysis of data is the development of a predictive, mechanistic model in which the process of hydrolysis is simulated. The comparison of experimental with simulated data will allow the researcher to identify which experimental data is as expected and which data is different from the expectations. Such alignment between approaches will benefit both the interpretation of experimental data as well as the fundamental understanding of the hydrolysis process.

In this study a mechanistic model is presented that allows for such predictive, quantitative simulation of the hydrolysis process of proteins. It is based on a stochastic (random) selection of substrate. It is now applied for the simulation of specific proteases (with 1 or 2 known cleavage sites), but can be easily adapted to describe the hydrolysis of non-specific proteases (2-10 cleavage sites), or enzymes that hydrolyze proteins based on a sequence of amino acids rather than specific cleavage sites.

Materials and Methods

Experimental data

Whey protein isolate (commercial Bipro) was hydrolyzed by *Bacillus licheniformis* protease (BLP), which is specific towards Glu and Asp residues [12]. Solutions of 1 % WPI were hydrolyzed by BLP at different pH of hydrolysis, pH 7.0, 8.0 and 9.0. Samples were taken at different degree of hydrolysis. The experimental details, methods for annotation and quantification of the hydrolysates obtained are described elsewhere (chapter 4). The data of experiments obtained in previous studies (chapters 4 and 6) including the enzyme selectivity towards each cleavage site, are used in this study to compare the results of the simulation model to experimental results.

MODEL

Design criteria

To allow optimal performance and usability, three different criteria were used in the development of the model to ensure that the model is:

1- Generic: The model should be set-up in a way that allows easy changes of the substrate, the enzyme (properties) and conditions (e.g. pH, enzyme to substrate ratio…). To achieve this, the model should not be built on information derived from a specific system.

2- Simple: The model should be based on as few assumptions as possible. This will reduce the risk for biased outcomes of the model.

3- Realistic: The model should have realistic input and output parameters, to function as a tool to understand and describe all experimentally determined parameters.

Based on these requirements the model was built using the following parameters:

Input parameters

1- The different proteins that are hydrolyzed. These are described by their primary amino acid sequence.

2- The abundance of each protein that is hydrolyzed.

3- The enzyme specificity is defined as the set of amino acids (cleavage sites) after which the peptide bonds are hydrolyzed.

4- Based on the sequences of the proteins, their abundances, and the enzyme specificity a maximal degree of hydrolysis (DH_{max}) is calculated based on the fraction f_i and the $DH_{max,i}$ of each protein i in the mixture.

(1) $DH_{max} = f_i \times DH_{max,\,i}$

The DH_{max} of each protein is calculated by dividing the number (#) of cleavage sites by the total number (#) of peptide bonds in the protein.

(2) $$DH_{max} = \frac{\#cleavage\ sites}{\#\ total\ number\ of\ peptide\ bonds}$$

This value is used to determine when the program should finish, for instance when 10, 50, or 100 % of the maximal DH is reached.

Output parameters

1- The total number of actions, which is an indicator that can later be used to translate to time. This is not done in this study, since it is not known if the total number of actions is proportional to the 'real' time of hydrolysis. It may, for instance, be that a correction needs to be included to account for the diffusion distance between enzyme and substrate. This value would then change as a function of the total number of molecules in the system.

2- The number of successful actions (i.e. the number of times the enzyme successfully hydrolyzes a peptide bond).

3- A list of all the different peptides that are formed at each point of the hydrolysis.

4- For each type of peptide, the number of molecules of this peptide that are present at each point of the hydrolysis.

5- The degree of hydrolysis, which is calculated from the total number of peptides ($\Sigma_{peptides}$), the length ($\#AA_{protein}$ - 1) and the number of initial proteins molecules ($\#proteins_{initial}$) (equation 3),

$$DH = \frac{\sum peptides_t - \#\, proteins_{initial}}{(\#\, AA_{protein} - 1) \times \#\, proteins_{initial}} \quad (3)$$

in which $\#AA_{protein}$ is the number of amino acids in the protein.

6- Based on the amount of each individual peptide at each point, the concentration of cleavage site products $C_{i,t}$ is calculated at each point of the hydrolysis using equation (4) (chapter 4).

$$C_{i,t} = \sum \left\{ C_{peptide}\left[x-y\right]_t \mid i = x-1 \cup i = y \right\} \quad (4)$$

$C_{i,t,}$ is the concentration of cleavage products formed at each time point t, after hydrolyzing peptide bond no. i, which equals the sum of all peptides of sequence [x-y], for which i = (x-1) or i = y.

7- To obtain an indication of the relative 'rate' of hydrolysis of each cleavage site, and therefore the selectivity, the concentration of cleavage site products at 90 % of DH_{max} was used. Experimentally, $C_{i,t}$ as function of time is used to determine the apparent cleavage rate of each individual cleavage site (chapter 4). This parameter was then used to determine the rate of selective hydrolysis and consequently the selectivity of the enzyme towards each cleavage site. Since in the model no real time is included, this approach could not be used.

Assumptions

The model is based on a stochastic process in which all molecules are randomly distributed over the system. In the basic model, the enzyme randomly selects any molecule, and subsequently randomly selects a position in this molecule.

All initially present proteins, and later on the peptides formed, are included in a hashmap. Basically, this hashmap is a matrix that contains a key (i.e. the peptide sequence) and a value for each type of peptide present. At the start, it contains one key for each type of intact protein molecule. Its value is the initial number of proteins. After

all initial proteins are hydrolyzed, this key will be present with value 0, and new keys will be added to represent the intermediate (peptides) and final products (peptides/amino acids) of the hydrolysis.
At each step of the hydrolysis, the program randomly selects one of the keys in the hashmap. This selection is weighted for the value of each key (i.e. the number of molecules) in the hashmap, to represent the random selection of one peptide or intact protein if the latter is still present, out of the whole mixture. The program then verifies whether or not the peptide selected (key) can be hydrolyzed. If the peptide can be hydrolyzed a random location in the peptide is selected to attack. If the chosen location (amino acid) satisfies the cleavage rules (e.g. if the chosen amino acid is defined in the enzyme specificity), a cleavage is made. This leads to the decrease of the value of the original key (peptide/intact protein) by 1, and the formation of two new peptides (keys). For each peptide that is already present, the value of the key corresponding to that peptide is increased by 1. This series of steps is continued until no more cleavable peptides are present in the hashmap. Apart from the choice of substrate, the current model, in its basic state, does not contain any other a-priori assumptions about the process of hydrolysis.

Options

In this first application of the model, relatively few options have been considered. One option that was tested was to change the manner in which the enzyme selects the substrate. In the basic model, the choice of substrate molecule and location on the amino acid chain sequence is completely random. As an alternative, the enzyme can also be programmed to choose only peptides that contain possible cleavage sites, and/or only those locations that satisfy the cleavage rules (i.e. enzyme specificity). An additional option that has been tested is the accessibility of the intact protein molecules (as explained below).
Additional options that are foreseen, but not applied in this study are:
1- Inclusion of the charge of the amino acid residues (and/or of total peptides) to vary the selectivity of the enzyme and the charges of the N and C terminal residues of the proteins/peptides.
2- Inclusion of factors for the accessibility of amino acids in the parent protein molecule based on the typical solvent exposure of such amino acids.
3- Extension of the model towards more a-specific enzymes.

Model structure

The simulation model was written in Java. The basic structure of the model is depicted in **figure 1**. Each run of the hydrolysis model will be further referred to as ‘a simulation’. The steps that are executed in each simulation are described by different modules in the program.

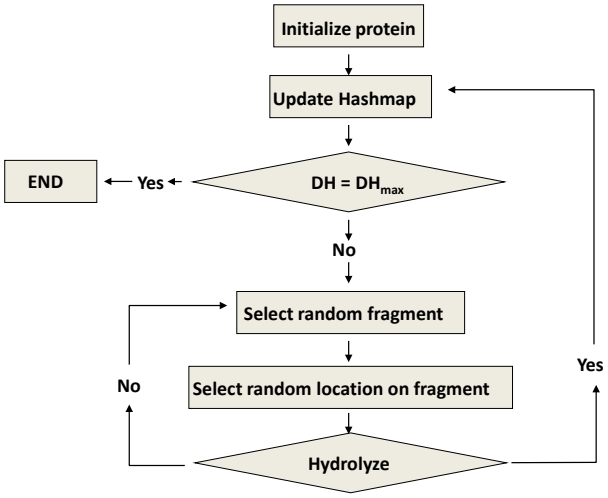

Figure 1. Schematic overview of the structure of the hydrolysis simulation model.

Initialize:

In this module the input parameters for the model are defined. These are the amino acid sequence(s) of the initial protein(s), initial number of protein molecules, enzyme specificity and/or selectivity, and DH_{max} at which the simulation should be stopped.

Protein: First, the amino acid sequence of the protein is converted into a 'protein molecule'. This protein molecule contains the code of each amino acid (single letter representation), the molecular weight, and pK_a of the side chain. In addition, other properties can be added, for later use in the program if needed (e.g. hydrophobicity).

Enzyme specificity: In this class the properties of the enzyme, or in other words the cleavage rule is defined. This can be the collection of amino acids after which the enzyme can hydrolyze the peptide bond (i.e. the enzyme specificity).

Enzyme affinity: In addition to the specificity, the affinity towards different cleavage sites can be defined. The affinity describes the chance for the enzyme to successfully hydrolyze a peptide bond. This may depend on different factors, such as the type of amino acid (e.g. Glu, or Asp). To do this, an additional random number is generated. If the number is equal to, or lower than the affinity towards the cleavage site, hydrolysis takes place. Otherwise the action is cancelled. An affinity of 1 consequently results in 100 % successful hydrolysis if this cleavage site is chosen. In a similar way, a modification can be made for the chance of the enzyme to hydrolyze a peptide bond in an intact protein compared to the hydrolysis of a bond in a fragment (peptide) resulting from previous hydrolysis steps. Such a modification would allow the evaluation of the two modes of enzymatic hydrolysis referred to as 'zipper' and 'one-by-one', described in the Linderstrøm-Lang theory [13]. Changes in the affinity towards different cleavage

sites will result in changes in the determined selectivity, which describes the relative rate of hydrolysis of the different cleavage sites.

Hydrolysis:
The hydrolysis will run from start until the required end-point (i.e. 90 % of the DH_{max}) is reached. In each step of the hydrolysis the enzyme will randomly select a substrate (as described above). In the case of successful hydrolysis, new peptides are formed and the hashmap will be updated.

Output:
After the hydrolysis is finished, the different output parameters are written to a CSV file format which can be opened in Excel. The output consists of:

- A header containing the initial settings of the hydrolysis.
- The data:
  the total number of actions (including unsuccessful attempts at hydrolysis),
  the total number of peptides at each point,
  the degree of hydrolysis,
  the number of molecules of each type of peptide at each point.
- The concentration of cleavage site products at each point during hydrolysis for all cleavage sites (for each substrate molecule).
- The total time needed for the simulation to reach completion.

To reduce the number of data points in the output file, the data is only recorded at required intervals (e.g. 0.01 % DH).

Simulations

1- *Testing the model.* First, a simple substrate (e.g. CAGAD) was defined to study the basic properties of the model. Due to the stochastic nature of the model, a relatively large number of initial peptides is needed to obtain smooth and reproducible results. For the simulations with the small peptide 100,000 initial molecules were used.

2- *Hydrolyzing intact proteins.* Intact β-lactoglobulin A (accession number P02754 in uniprot) was used as substrate in the model to see the differences in behavior between a 'real' sequence and a sequence in which the cleavage sites are homogeneously distributed. The sequence of β-lactoglobulin A is: "*LIVTQTMKGLDIQKVAGTW YSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENDECAQKKIIAEKTKIPAVFKIDALNENK VLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQC HI*." For the simulations 10,000 initial molecules were used.
To compare with a theoretical protein for which the amino acids E (cleavage sites) are at regular intervals, the sequence "*AAAAAAAA**E**CCCCCCCC**E**FFFFFFFF**E**GGGGGGG G**E**HHHHHHHHH**E**IIIIIIII**E**KKKKKKKK**E**LLLLLLLL**E**MMMMMMMM**E**NNNNNNNN**E**PPPPPPPP**E**QQQQQ QQQ**E**RRRRRRRR**E**SSSSSSSS**E**TTTTTTTT**E**VVVVVVVV**E**WWWWWWWW**E**YYYYYYYY*" was used. For the simulations 10,000 initial molecules were used.

To compare the selectivity obtained by simulation to the selectivity obtained experimentally, a rescaled relative selectivity was calculated. The selectivity in both sets of data is first rescaled by subtracting the lowest selectivity value to all values. The rescaled relative selectivity is calculated using the highest rescaled selectivity as 100 %.

3- *Hydrolysis of multiple proteins.* To further illustrate the potential use of the model, simulations were performed with an initial protein composition that mimics the composition of the industrial whey protein isolate (Bipro). The initial numbers of protein molecules were 4900, 3300, and 1800 for β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin, respectively (based on the composition of Bipro as determined previously (chapter 4)). The sequence of β-lactoglobulin A and B (accession number P02754 in uniprot) and of α-lactalbumin (accession number P00711) were used. These simulations were run without any further assumptions on the enzyme selectivity.

4- *Changing the accessibility of intact proteins.* Previously, it was described that by changing the pH of the solution, a higher proportion of remaining intact protein was observed (at any DH) upon lowering the pH from 9.0 to 7.0 (chapter 6). This was interpreted as a decreased ability of the enzyme to hydrolyze the intact protein (i.e. decreased accessibility of the substrate). To simulate the effects of a decreased accessibility of intact proteins (P), the chance to hydrolyze intact proteins (compared to the hydrolysis of derived peptides) was varied in the model. To do this, after the selection of the cleavage site - and identifying the cleavage site to be in agreement with the enzyme specificity, a new random number was generated. If the resulting random number was lower than the chance to hydrolyze the intact protein, the hydrolysis occurred. Otherwise, the enzyme would continue to select a new substrate and a new location on the substrate. For these simulations accessibilities of the intact protein (P) of 1, 0.7 and 0.3 were used, where 1 indicates 100 % accessibility. The system consisted of the same initial molecules described above for the whey protein isolate. The simulated data of peptides formation are expressed in relative number, which means that the initial number of protein molecule is defined as 100 %.

5- *Changing the affinity towards certain cleavage sites.* Two different approaches were used to change the affinity towards different cleavage sites. Since it is known for instance that BLP has a 1000 times lower selectivity for Asp than for Glu (chapter 4), a method was defined to include different affinities towards Asp and Glu. Similarly as with the accessibility, a random number was used. When the value was lower than the affinity towards the cleavage site, the hydrolysis was considered successful. For a more detailed assignment of affinities to each individual cleavage site, an array of values was used. In the same way, the value of a random number, compared to the assigned affinity determined if hydrolysis occurred. When 1000 times lower values for Asp than for Glu were used for the affinity, a disproportional response was observed in the simulation. For this reason, it was decided to reduce the affinity towards certain

cleavage sites by 10 and 50 % using the values 0.9 and 0.5 as affinity. The experimental and modeled selectivities towards individual cleavage sites were compared on relative scales. For that, the relative selectivity was calculated by setting the highest selectivity determined for each individual cleavage site to 100 %.

Results and Discussion

Testing the model

To demonstrate the basis of the model a first test was made to simulate the hydrolysis of a small peptide, GACAD hydrolyzed after amino acid A. (**figure 2**) This shows directly the different kinetics of formation and breakdown of the different peptides. The C- and N- terminal peptides without further possible cleavage sites ([D] and [GA]) are formed directly from the start of the hydrolysis.

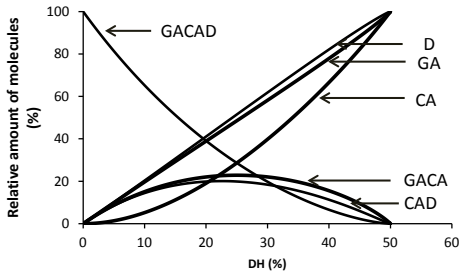

Figure 2. Simulation of hydrolysis of 100,000 molecules of [GACAD] using an enzyme that cleaves after 'A'.

It can be seen that the two intermediate peptides, which still contain a cleavage site, [GACA] and [CAD] are present at roughly equal concentration. In addition, the peptide [CA], which can only be formed after cleavage of two cleavage sites, is formed at a slower rate than [D] or [GA]. The data showed a surprising result. The total number of actions (successful and unsuccessful tries for hydrolysis) was varied between repeated simulations. (results not shown) The standard error of the total number of actions was around 2 %. The reason for this variation is the stochastic nature of the hydrolysis process. In the final stages of the hydrolysis a large number of peptides are present and only a small number of possible cleavage sites are remaining. Since both the molecule and the amino acid where the enzyme tries to hydrolyze a peptide bond are chosen randomly, the chance to find the last cleavage site is very small towards the end of hydrolysis. Consequently, the exact end-point where 100 % of the maximal degree of hydrolysis is reached varies slightly between simulations.

Another observation is that the kinetics for the N- and C-terminal peptides is not exactly identical, even though the peptide seems symmetric. A closer look shows that there is indeed a difference. The intermediate peptide [GACA] consists of 4 amino acids, while the other intermediate peptide [CAD] only contains 3 amino acids. Consequently, the chance for [GACA] to be hydrolyzed (when chosen) is 1/4, while the chance for [CAD]

to be hydrolyzed is 1/3. To test if this difference indeed explains the observed differences in hydrolysis kinetics, the same simulation was performed with the peptide [GACADE] where the two intermediate peptides [GACA] and [CADE] have the same length. (**figure 3**) The results show indeed that in this case the kinetics of the two intermediate peptides, as well as the C- and N- terminal peptides are now identical.

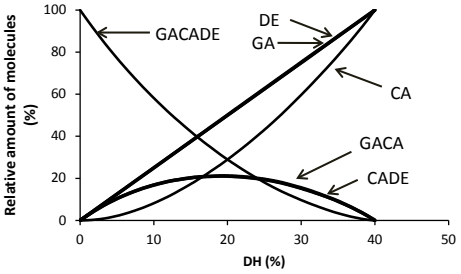

Figure 3. Simulation of hydrolysis of 100,000 molecules of [GACADE] using an enzyme that cleaves after 'A'.

In a previous study, the concentration of cleavage site products was used as an indication for the selectivity of the enzyme (chapter 4). The selectivity was expected to be mostly due to differences in the charge of peptides and accessibility of the substrate. The above results strongly suggest that there is already a change in the relative rate of hydrolysis of cleavage sites that is related to the sequence of the peptide. More precisely, there is an effect of the distribution of the cleavage sites over the peptide sequence. This is further illustrated by plotting the concentration of cleavage site products (for the cleavage sites A2 and A4) as function of the DH. (**figure 4**)

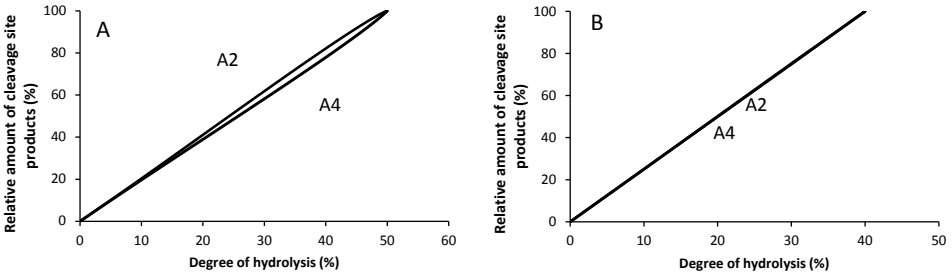

Figure 4. Relative amount of cleavage site products of A2 and A4 as function of the DH for peptide (A) [GACAD] and (B) [GACADE].

The increase in the concentration of cleavage products as a function of time was previously used to determine the apparent cleavage rate of each cleavage site and subsequently the selectivity (chapter 4). Since the model does not have a parameter for time, the increase of the concentration of cleavage site products as function of DH can be used as an indication of the selectivity. In this way, the selectivity towards A2 and A4

in [GACAD] is 51 and 49 % respectively, while it is exactly 50 % for both cleavage sites in peptide [GACADE].

Hydrolyzing intact proteins

Based on the hydrolysis of the small peptide, it was found that the distribution of cleavage sites over the protein may influence the rate of hydrolysis of each individual cleavage site. To test this, a simulation was run with the sequence of β-lactoglobulin A and a similarly large protein with a regular distribution of cleavage sites (see materials and methods section). (**figure 5**) In this simulation the enzyme was set to be specific for 'E', since the BLP used in the experimental work is specific for E (glutamic acid).

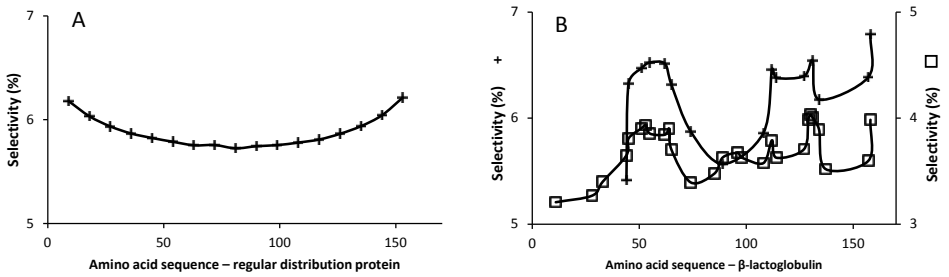

Figure 5. Simulation of the selectivity for the different cleavage sites for (A) a theoretical protein with cleavage sites at regular intervals and (B) β-lactoglobulin A using an enzyme that hydrolyzes Glu only (+) and Glu and Asp residues (□).

For the regularly distributed protein, the selectivity for the cleavage sites shows a parabolic curve. The selectivity towards the cleavage sites closest to the N- and C-terminal ends of the protein sequence is the highest with a value of 6.2 % and the selectivity is the lowest in the center of the sequence with a selectivity of 5.7 % (decrease of 8 %). (**figure 5A**) For the real protein (β-lg A) sequence, a more diverse distribution of selectivity is found. Surprisingly, the cleavage site closest to the N-terminal end (Glu-44 or Asp-11) has a very low selectivity. Some other parts of the sequence (i.e. 45-65, 112-131 and 158) have a relatively high selectivity. (**figure 5B**) Apparently, this is the effect of the distribution of cleavage sites over the protein sequence. Areas where cleavage sites are relatively close together are hydrolyzed faster. Experimentally, cleavages sites 45, 55, 62 and 158 were found with a high selectivity, which is in agreement with the predicted data. (**table 1**) In contrast, cleavage sites 51, 65, 112, 114, 127 and 131 had a lower experimental selectivity than in the simulation.

Based on the observations from the simulation model, it needs to be noted that the selectivity (i.e. the relative rate of hydrolysis of each cleavage site) is determined not only by the accessibility and the enzyme preference, but also by the location of the cleavage site in the parent protein molecule.

Table 1. Comparison of the rescaled relative selectivity towards the different cleavage sites determined experimentally and in the model.

	model (E)	model (E+D)	exp
β-lg cleavage sites	Rescaled relative selectivity (%)		
11	-	52	0.02
28	-	54	0.03
33	-	62	0.16
44	0	75	0
45	**66**	**86**	**100**
51	77	91	2.35
53	-	93	0
55	**81**	**88**	**88**
62	**81**	**85**	**82**
64	-	0	0
65	66	77	7.06
74	33	60	35
85	-	66	0.13
89	13	75	35

	model (E)	model (E+D)	exp
β-lg cleavage sites	Rescaled relative selectivity (%)		
96	-	78	0.06
98	-	75	0
108	36	74	41
112	76	86	0
114	72	77	7.65
127	73	81	29
129	-	98	0
130	-	100	0
131	82	99	0.21
134	57	92	94
137	-	70	0.09
157	69	76	12
158	100	97	47

Hydrolysis of multiple proteins

One important property of models is the flexibility to be applied to different systems. To show the flexibility of the model, the industrial protein isolate Bipro was used, containing mostly β-lactoglobulin A and B, and α-lactalbumin (chapter 4). In the experimental results, a relatively slow hydrolysis of α-lactalbumin was observed compared to β-lactoglobulin. A simple simulation of the hydrolysis of whey protein isolate (WPI) with an enzyme that only hydrolyzes after Glu residues showed basically that indeed β-lactoglobulin was hydrolyzed faster than α-lactalbumin. (**figure 6**)

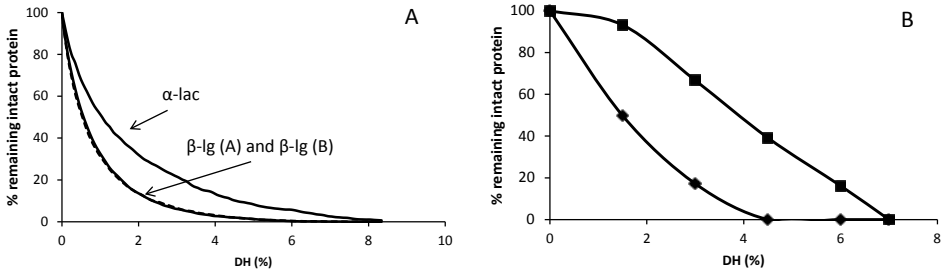

Figure 6. (A) Simulation of the proportion of remaining intact protein for the hydrolysis of WPI (Glu specific) and (B) experimentally determined proportion of remaining intact protein (◆) β-lactoglobulin and (■) α-lactalbumin as a function of DH for hydrolysis of 1 % WPI by BLP (chapter 4).

Although there is a profound difference in the shape of the curve, α-lactalbumin is indeed hydrolyzed at a slower rate. This confirms the conclusion of a previous study (chapter 4) that the slower decrease of the amount of α-lactalbumin with increasing DH of the total protein isolate is (at least partly) related to the low concentration of the protein in combination with the low number of cleavage sites. However, the decrease of both proteins with increasing DH is still faster in the simulation than in the experiment.

This may be due to the fact that experimentally, the intact protein molecule is not fully accessible to the enzyme. To include this factor, additional simulations were performed.

Changing the accessibility of intact proteins

Hydrolysis of the intact protein

*S*imulations were performed with the WPI, for which the accessibility of (all) intact proteins was varied from 1 (or 100 %) to 0.1, while keeping the affinity towards each cleavage site at 1. (**figure 7**) As expected, by decreasing the accessibility of the intact proteins, a higher proportion of remaining intact β-lactoglobulin at each DH is observed. The resulting trends of proportion of remaining intact protein correspond to the trends observed in the experiments where the pH of the hydrolysis was decreased (chapter 6). This supports the idea that the experimental observations may be due to decreased accessibility of intact proteins with decreasing pH.

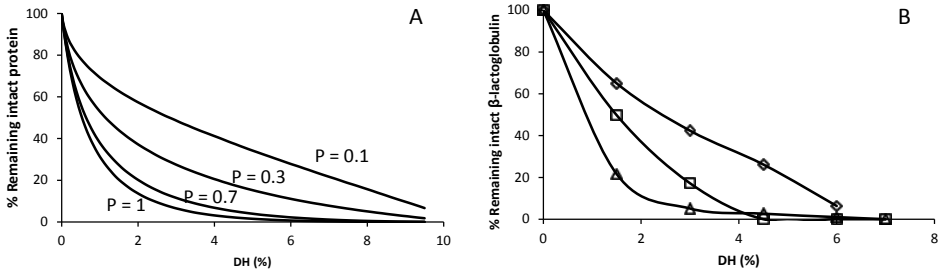

Figure 7. (A) Simulation of hydrolysis of intact β-lactoglobulin as a function of DH for different accessibility (P) of the intact proteins set and (B) experimental data of the change in hydrolysis of intact β-lactoglobulin as function of DH by change in pH, (◇) pH 7, (□) pH 8, (△) pH 9 (chapter 6).

By changing the accessibility (P) towards all intact proteins in WPI to a lower value (i.e. P = 0.3), the simulation data for β-lactoglobulin are more comparable to the experimental data. (**figures 8 and 6B**) Still, the predicted data of the proportion of remaining α-lactalbumin do not correspond exactly to the experimental data. These simulations can be used to determine if the changes in accessibility also influence the peptide release kinetics.

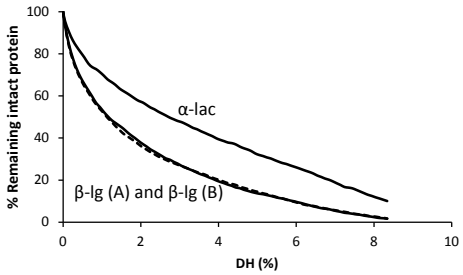

Figure 8. Simulation of the proportion of remaining intact β-lactoglobulin (A and B) and α-lactalbumin for the hydrolysis of WPI (Glu specific) with accessibility P = 0.3.

Kinetics of peptide formation

In previous experimental analysis of the hydrolysates (chapters 4, 5 and 6) it was first considered that all possible peptides should be present at one time during the hydrolysis. However, while 84 different peptides were found in the hydrolysates of WPI (chapter 6, **Annex 2**), 658 peptides are expected based on the proteins and the enzyme selectivity of BLP (Glu, Asp). From the simulation of the hydrolysis of the theoretical regular protein, which has cleavage sites at regular intervals, it is clear that even in the simulation not all peptides are formed at equal concentrations. The concentration of each peptide was found to be strongly dependent on the number of cleavage sites present in the peptide. (**figure 9**) This indicates that peptides with high number of cleavage sites (e.g. 16, 12) do not accumulate and have a rate of formation comparable to their rate of degradation. Peptides with less cleavage sites (e.g. 2, 3) accumulate more, which means that their rate of formation is higher than their rate of breakdown. This observation may also explain why only a limited number of peptides is annotated and quantified in the experiments. Based on this, it is considered that most of the peptides formed, especially the longer peptides, will be present in very low concentrations. More specifically, in the simulation at DH=7 %, for instance, 20 % of the peptides account for 90 % of the total amount of peptides.

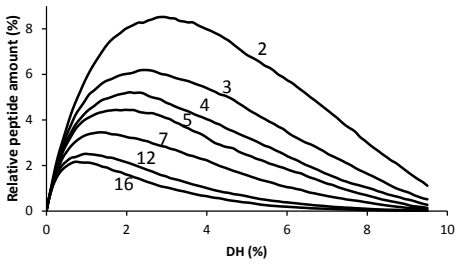

Figure 9. Relative amount of peptides as a function of DH for the theoretical regularly ordered protein with cleavage sites at regular intervals. Numbers in the graph indicate the number of cleavage sites in the peptides formed.

In the experimental analysis, up to 84 different peptides were annotated and quantified as a function of the DH (chapter 6). Three different types of behavior were observed as function of DH (and time). (**figure 10**) The first type of behavior corresponds to intermediate peptides, which can be formed from one cleavage on the intact protein e.g. β-lg[1-45] and β-lg[135-162]. Their concentration are first increasing, corresponding to the formation of the peptides. The peptides are then further hydrolyzed. The second type of behavior is shown by peptide β-lg(A)[109-127], which is first slowly increasing in concentration and then decreasing. This peptide is an intermediate peptide. It is obtained from at least two cleavages on the intact protein and then further hydrolyzed. The third type of behavior corresponds to final peptides, e.g. β-lg(A)[115-127], which do not contain any cleavage site and are only increasing in concentration.

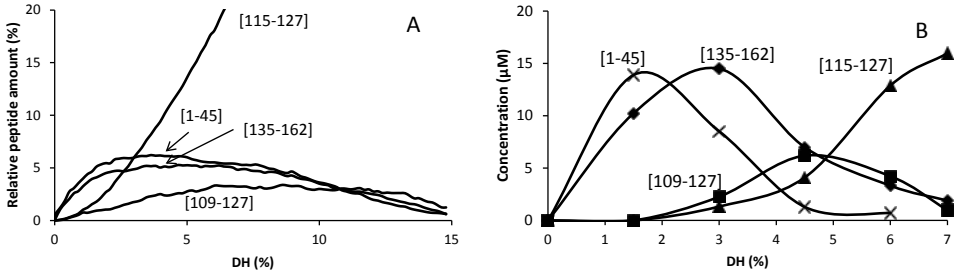

Figure 10. (A) Simulation of kinetics of peptide formation and (B) experimentally determined concentration (µM) of different peptides resulting from hydrolysis of β-lactoglobulin as a function of DH.

As a comparison, the model was run using the composition of WPI (and same affinity towards E and D residues with P = 0.7). The results for four peptides used as examples representative for the three types of behavior are quite similar in the model as in the experimental data. (**figure 10**) The important parts are the maximum observed for β-lg[135-162] and β-lg[1-45]. In the experimental data, the curves are sharper and have a clearer maximum. Another important observation is the relatively low amount of peptide β-lg(A)[109-127] both experimentally and in the model. It was experimentally found that the enzyme selectivity is rather low for the cleavage sites 115 and 127 while in the model the affinity was set to be the same for all cleavage sites. This might explain why peptide β-lg(A)[115-127] is found at a lower concentration and at a higher DH experimentally than in the simulated data.

Simulation based on selectivity determined experimentally

The model showed promising features, describing to some extent the basic behavior and complexity observed in the experiments. Since in a previous study the selectivity of the enzyme towards the different cleavage sites in β-lactoglobulin was determined (chapter 4), a simulation was performed with the (normalized) experimental selectivities. It was found that the simulated data did not agree with the experimental data. (data not shown) In addition, the relative selectivity towards the cleavage sites obtained from the simulation did not agree with the initially set values. This discrepancy is due to the fact that the real selectivity (determined from the peptide release kinetics) is not only determined by the accessibility towards the intact protein, but also by the distribution of the cleavage sites. This makes it difficult to recalculate the observed experimental selectivities towards a value that should be used as affinity to the cleavage site in the model.

By changing the accessibility towards the intact protein (P), different maximum concentrations of the peptides are reached. (**figure 11)**

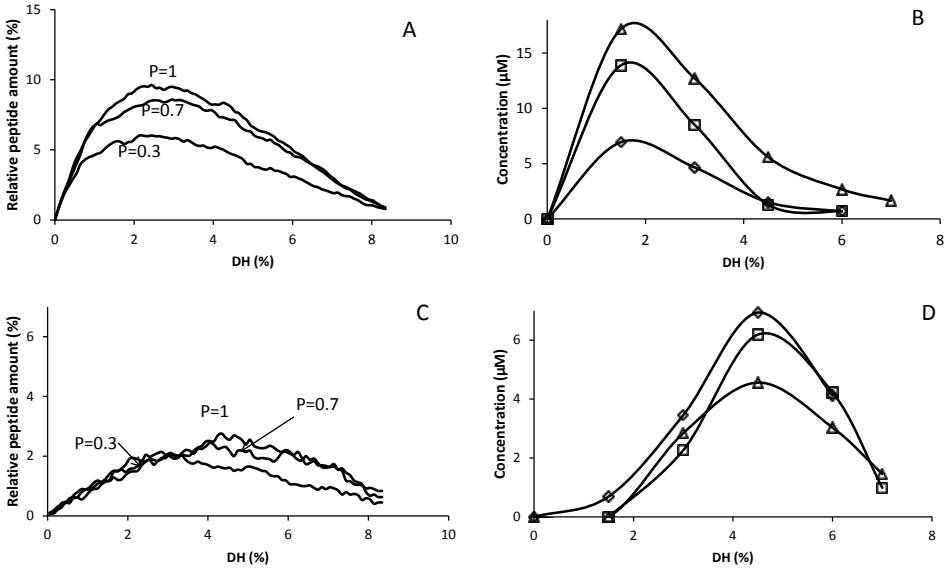

Figure 11. Concentration of peptide β-lg[135-162] (A) model with different accessibility (P) for the intact protein and (B) experimentally obtained data at different pH of hydrolysis (◇) pH 7, (□) pH 8, (△) pH 9 (chapter 6). Concentration of peptide β-lg(A)[109-127] (C) model with different accessibility (P) for the intact protein and (D) experimentally obtained data at different pH of hydrolysis (chapter 6).

Typically, the differences in experimental data due to the different pH of hydrolysis applied can be matched by varying the accessibility of the proteins in the simulation model. A decrease in the maximum concentration is observed with decreasing accessibility towards intact protein. This corresponds to the experimental data, at decreasing pH, for peptide β-lg[135-162]. (**figures 11A and B**) For this peptide, a shift in the maximum concentration is observed for the simulated data. The maximum concentration is reached at a lower DH for the lower accessibility. In contrast, experimentally, the maximum concentration is reached at the same DH for the three pH of hydrolysis.

For peptide β-lg(A)[109-127], again the trend of increasing concentration followed by decrease in concentration observed in the model is similar to that observed in the experiment. (**figure 11C and D**) In the model, the maximum concentration is reached at lower DH with decreased accessibility, indicating a faster degradation of the peptides for P = 0.3. Experimentally, the maximum concentration is reached at the same DH. Hence, for this peptide, the simulated data do not agree as well as those for β-lg[135-162]. The reason for lower agreement between experimental and simulated data may be due to the fact that the cleavage sites 108, 115 and 127 were found experimentally to have a low selectivity. As a consequence, in the model the peptide β-lg[109-127] is degraded faster than in the experiment.

Determination of the selectivity

As a preliminary experiment, the changes in selectivity are determined for different accessibilities towards intact proteins in WPI. By decreasing the accessibility towards the intact protein, the selectivity of cleavage sites with high selectivity (at P = 1) is decreased, while the selectivity towards cleavage sites with initial low selectivity (at P = 1) is increased. Overall this leads to smaller differences in the selectivity between the different cleavage sites. (**figure 12**)

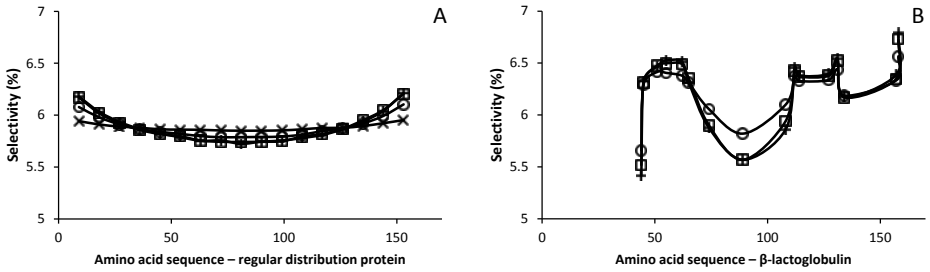

Figure 12. Simulation of the selectivity for the different cleavage sites for (A) a theoretical protein with cleavage sites at regular intervals and (B) β-lactoglobulin A with different accessibilities towards the intact protein (+) P = 1, (□) P = 0.7, (○) P = 0.3 and (x) P = 0.1.

Changing the affinity towards certain cleavage sites

Cleavages after Glu

Several parameters in the model using WPI were adjusted to compare the selectivity of each cleavage site to the corresponding selectivity obtained experimentally. At first, data were modeled for only Glu cleavage sites with an affinity to 1, with different accessibilities of the intact protein (β-lactoglobulin A and B and α-lactalbumin) to be hydrolyzed. The affinity describes the chance for the enzyme to hydrolyze a peptide bond. For 9 cleavage sites after Glu, the experimental data are rather correctly predicted as shown with two examples. (**figures 13A and 13B**)

For the other 6 cleavage sites after Glu residues, the model shows a completely different trend than observed with the experimental data as observed for example for cleavage sites 127 and 134. (**figures 13C and 13D**) There seems to be no correlation with the position of the residue in the primary sequence or with the surrounding amino acids to explain the differences between model and experimental data.

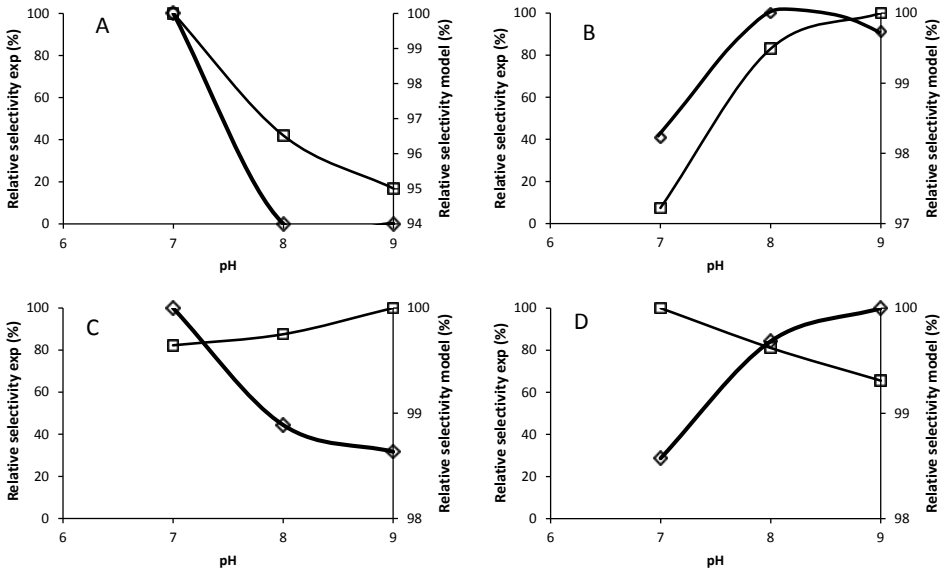

Figure 13. Examples of selectivity towards the cleavage sites (A) 44; (B) 158; (C) 127; (D) 134. Simulation when only Glu residues are cleaved with an affinity of 1 for all cleavage sites Glu. (◇) experimentally determined relative selectivity (chapter 6) and (□) modeled relative selectivity. Relative selectivity determined using the highest selectivity of each cleavage site as 100 %.

Based on these preliminary observations, the affinity for certain cleavage sites after Glu residues was decreased. This was based on selectivity determined experimentally. It was indeed found that the enzyme has a high and intermediate selectivity towards 9 cleavage sites after Glu and a low or very low selectivity towards the 7 other Glu cleavage sites in β-lactoglobulin (chapter 4). To mimic the effect, the affinity of the enzyme towards these 7 cleavage sites with lower selectivity was set to the arbitrarily chosen values 0.9 and 0.5. (**figure 14**)

Changing the affinity for 7 out of 16 cleavage sites does not result in one single effect. For some cleavage sites, the prediction was still comparable to the experimental data. (**figure 14A**) In other cases, the changes in affinity did not improve or actually made a prediction further away from the experimental data. (**figures 14B and 14C**) For again other cases an improvement in the prediction was seen, e.g. cleavage site 134. (**figure 14D**)

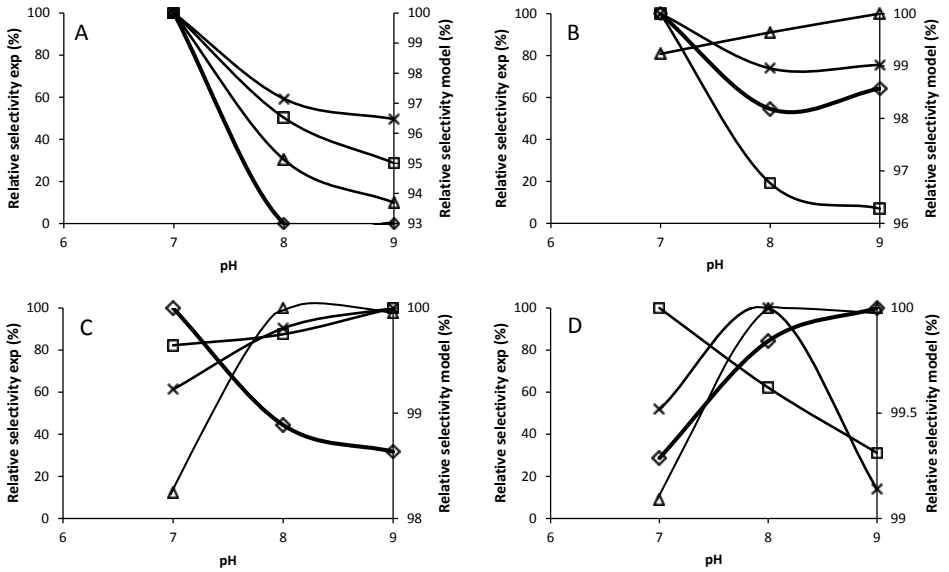

Figure 14. Examples of selectivity towards the cleavage sites (A) 44, (B) 74, (C) 127 and (D) 134. Simulations including Glu residues as cleavage sites at different affinity: (□) affinity of 1 for all cleavage sites Glu, (X) affinity of 0.9 towards 7 Glu residues and 1 for the other 9 Glu, or (△) affinity of 0.5 for 7 Glu residues and of 1 for the other 9 Glu. (◇) Experimental relative selectivity (chapter 6). Relative selectivity determined using the highest selectivity of each cleavage site as 100 %.

Cleavages after Glu and Asp

It has been determined that the enzyme is specific for Glu and Asp residues [12]. In addition, it has been determined experimentally that the enzyme has a lower selectivity towards Asp residues than Glu residues (chapter 4). To try to predict more accurately the selectivity of the enzyme towards the different cleavage sites, an affinity of the enzyme was also set towards Asp residues. (**figures 15 and 16**) In addition, to obtain an accurate simulation, a lower affinity was set for Asp residues than for Glu residues. The affinity towards all Glu residues was set to 1 while the affinity for Asp residues was either 0.9 or 0.5.

In these scenario, there are again several types of behavior. Firstly, including Asp residues at either of the affinities has no influence on the quality of the prediction for cleavage site 158, for instance. (**figure 15B**) This might be because no aspartic acid residues are in the neighborhood of the cleavage site 158. Secondly, for cleavage site 44 the quality of the prediction decreases with a decrease in affinity towards Asp residues. (**figure 15A**) Thirdly, when Asp residues are included the prediction of cleavage site 134 corresponds to the experimental data. This is an improvement compared to the first scenario in which only Glu residues were included. (**figure 15D**) Finally for cleavage site 127, the simulated data do not match the experimental data. (**figure 15C**)

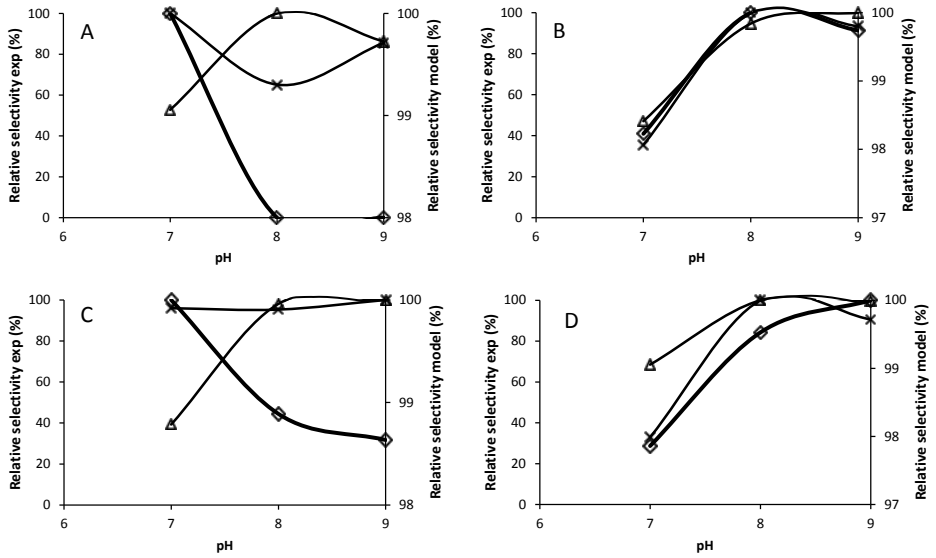

Figure 15. Glu cleavage sites (A) 44, (B) 158, (C) 127 and (D) 134. Data simulated for an enzyme specific towards Glu and Asp with different affinities for Asp, (X) affinity 0.9, (△) affinity 0.5. (◇) experimental data (chapter 6). Relative selectivity determined using the highest selectivity of each cleavage site as 100 %.

With respect to Asp residues, for 4 cleavage sites the model and the experimental data are comparable. (e.g. cleavage site 28, **figure 16A**) For 3 other cleavage sites after Asp, an opposite behavior is seen between simulated and experimental data. (e.g. cleavage site 11, **figure 16B**) In general, for Asp residues the quality of the prediction is increased with affinities towards Asp decreased to 0.5. For Glu cleavage sites, however, the decreased affinity towards Asp residues result in a decreased quality of the prediction when compared to the experimental data. This was for instance observed for cleavage site Glu-44 (**figure 15B**), which is in the neighborhood of cleavage sites Asp-28 and Asp-33. This shows the influence of the neighboring cleavage sites and their selectivity on the selectivity of each cleavage site.

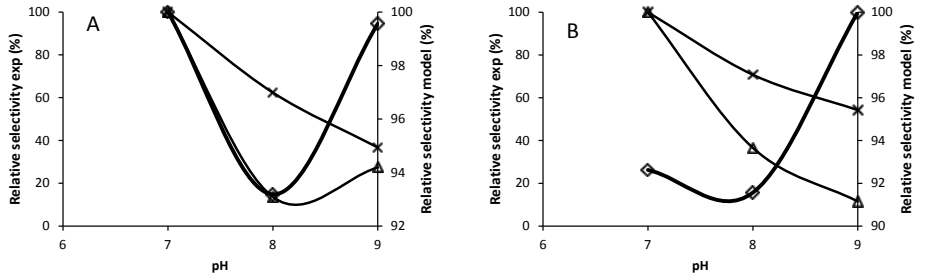

Figure 16. Asp cleavage sites (A) cleavage site 11 and (B) cleavage site 28. Data simulated for an enzyme specific towards Glu and Asp with different affinities for Asp, affinity (X) 0.9, (△) affinity 0.5. (◇) experimental data (chapter 6). Relative selectivity determined using the highest selectivity of each cleavage site as 100 %.

Conclusions

Using the developed stochastic model, a quite good agreement between simulated and experimental selectivity was observed for most of the cleavage sites. Still, the fact that the selectivity cannot be predicted easily for all cleavage sites shows the complexity of the mechanism of hydrolysis. It was shown that the selectivity depends on the accessibility of the intact protein, the distribution of the cleavage sites in the sequence and the affinity of the enzyme towards each cleavage site. There is also an influence of the affinity towards the neighboring cleavage sites on the experimentally determined selectivity. Further simulations can be performed to predict the conditions of formation of specific peptides.

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Chapter 9

General Discussion

At the start of this PhD project it became apparent that there was a lack of methods and vocabulary to describe the hydrolysis process and the influence of substrate concentration on the enzymatic protein hydrolysis. Firstly, there is not one single method to unambiguously describe the kinetics of the hydrolysis. Secondly, there was no established method to fully describe the peptide composition of the hydrolysates. In addition to these challenges, the explanation of the observed effects of increased substrate concentration was also not straightforward. At high concentrations (i.e. > 10 % (w/v)) the properties of protein solutions (e.g. conductivity) deviate from the behavior in (ideal) dilute systems. The aim of this PhD project was thus to describe the peptide composition and the hydrolysis mechanism and to develop the methods needed to reach a complete description. With these methods, the influence of varying system conditions (e.g. substrate concentration and pH) on the mechanism of hydrolysis was studied.

In chapter 4, selectivity was introduced as a new quantitative parameter to characterize the mechanism of hydrolysis. It describes the relative rate of hydrolysis by the enzyme of each individual cleavage site in the total protein. With this newly developed parameter, the questions raised at the beginning of this study can be answered. It was shown that not all cleavage sites within a protein are hydrolyzed equally by the enzyme. Large variations in the selectivity from 0.003 % to 17 % were observed (chapter 4). In addition, it was shown that increasing the substrate concentration results in a decrease in the overall hydrolysis rate as well as in the DH reached (chapter 2). Furthermore, it was concluded that the mechanism of hydrolysis as determined by the selectivity is influenced by increasing substrate concentration. A large influence in the selectivity towards the four cleavage sites for which the enzyme has the highest selectivity was observed at increasing protein concentration. The selectivity is increased by a factor 2 for two of these four cleavage sites while it was decreased by a factor 2 for the other two cleavage sites at 10 % (w/v) whey protein isolate (WPI) compared to 1 % (w/v) (chapter 5). In addition other changes as a function of the substrate concentration are observed for cleavage sites with lower selectivities. In terms of absolute concentrations these latter effect may not influence the overall composition of the hydrolysate. Nevertheless, if such cleavage sites involve the products of highly active bio-active peptides, also these effects will be of interest. These conclusions were based on the complete analysis of the peptides present in the hydrolysates obtained during enzymatic hydrolysis. Before reaching these conclusions, a number of practicalities and challenges had to be considered and dealt with. The first consideration is the quantification of enzyme activity and its use to compare different enzymes or substrates. Secondly, difficulties in sample handling, due to gelation occurring during hydrolysis of highly concentrated protein solutions. The third point addressed is the effect of enzyme inactivation on the composition and properties of the hydrolysates. Finally, the parameters, developed to assess the quality of peptide identification and quantification, are introduced. These challenges are exposed in the next sections of this chapter along with more detailed conclusions on the outcomes of the project.

Determination of the protease activity

The first event in an enzymatic protein hydrolysis process is the addition of enzyme to the protein solution. To compare hydrolyses, the systems should be standardized to a certain extent. For enzymatic hydrolyses performed with different enzymes, the standardization is typically based on the activity (g x U/g) of the enzyme preparation, rather than on the total weight, since the enzyme preparations used may contain other proteins, or carbohydrates or salts, next to the enzyme. The enzyme activity is generally defined as the rate at which a substrate is consumed or the rate at which products are formed during enzymatic incubation. One enzyme unit U is defined as the amount of enzyme which will catalyze the conversion of one µmole of substrate per minute under given conditions. While this definition seems simple, the fact that for proteases different activity assays with different types of substrate are available indicates that the use of the unit is not unambiguous.

Protease activity assays are generally based on colorimetric evaluation of product formation or substrate consumption, using either synthetic peptides, or (labeled) proteins. The azocasein assay, for instance, uses the hydrolysis of a labeled protein. The activity is calculated from the release of peptides containing the label in the solution after 10 minutes at a defined pH and temperature of hydrolysis, after precipitating the intact protein. While the activity determined using this assay is valid for this specific substrate, it may not be the same for other substrates. Between substrates a number of parameters can change, such as the number of bonds that can be potentially cleaved and the accessibility to these bonds. The latter depends on the conformation of the protein. In the same way, to compare the activity of two enzymes, the choice of the substrate is important. For two enzymes with different specificities, the number of potential bonds to cleave on the model substrate will be different. Hence this makes it difficult to find one substrate to compare two enzymes with different specificities.

To illustrate the difficulties in defining the protease activity, additional data collected during the project are presented here. The activity of two enzymes with the same specificity was determined by the azocasein assay. (**table 1**) The two enzymes used were BLP (*Bacillus licheniformis* protease, (NS-37005)), obtained from Novozymes (Bagsvaerd, Denmark) and the enzyme V8 from *Staphylococcus aureus* (#70213122) obtained from Roche (Almere, The Netherlands). Both enzymes are specific for Glu and Asp residues and have been used in previous studies for the hydrolysis of whey proteins [1,2].

The activity as determined by the azocasein assay was expressed in increase of absorbance unit (AU) per time and per mg enzyme [3]. The activity were found to be 0.26, and 3.0 $AU/mg_{enz}/min$ for BLP and V8, respectively. In addition to the standard assay, the hydrolysis of azocasein was also monitored using the pH-stat method. In contrast to the results of the standard assay, the activity determined from the initial slope of the degree of hydrolysis (DH) versus time curves, was similar for both enzymes: 0.02 s^{-1}. (**table 1**)

Table 1. Activity of BLP and V8 towards azocasein determined by azocasein assay and with the pH-stat method and for β-lactoglobulin determined by the pH-stat method.

Substrate	Azocasein		β-lg	
Enzyme	BLP	V8	BLP	V8
Assay (AU/mg_{enz}/min)	0.26	3.0		
pH-stat initial rate (s^{-1})	0.02	0.02	0.01	0.0003

The activity determined by the standard azocasein assay actually shows the loss of intact protein. Consequently, the comparison of the two enzymes with this assay is as much a comparison of the mechanism of hydrolysis as a comparison of the activity. Since for both enzymes the initial part of the DH versus time curve (obtained by the pH-stat method) was similar, this shows that V8 has a higher activity towards intact azocasein than BLP.

Since the enzyme activity may also depend on the substrate, both enzymes were also tested on β-lactoglobulin as a substrate in the pH-stat. On this substrate, BLP has a higher activity than V8 as determined by the initial rate on the DH curves. (**table 1**) The activity determined from the initial rate is again different from the results determined with azocasein as substrate. This confirms results obtained in a previous study showing a higher rate of hydrolysis on β-lactoglobulin with BLP than for V8 using the pH-stat [4]. (**figure 1A**) In that experiment, similar activities of enzyme, based on azocasein assay were used to hydrolyze the β-lactoglobulin. This study also showed that V8 had a lower affinity towards intact β-lactoglobulin than BLP. (**figure 1B**)

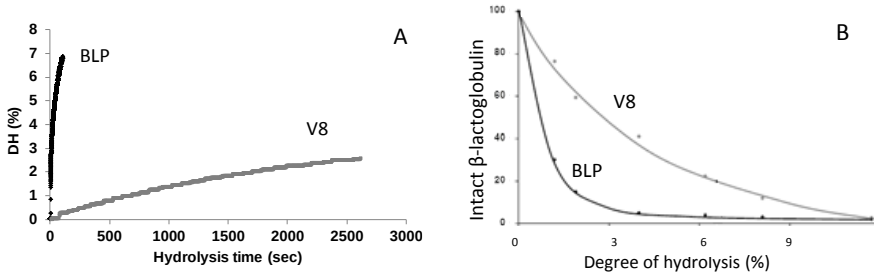

Figure 1. (A) Hydrolysis curves and (B) Proportion of remaining intact β-lactoglobulin as a function of the degree of hydrolysis for hydrolysis of 0.5 % (w/v) β-lactoglobulin by BLP and by V8. Adapted from [4].

These experiments show that depending on the analysis method and substrate, BLP had either a higher, an equal, or a lower activity than V8. A similar observation has been made for two a-specific protease preparations both containing Subtilisin as the main component, that were compared in two assays [5]. In the azocasein assay, Alcalase 2.4 had a higher activity than Prolyve 1000 while on another substrate (succinyl-Ala-Ala-Phe-paranitroanilide) Alcalase had a lower activity than Prolyve. The activity values, determined by azocasein, were used to standardize the hydrolysis of

WPC. Still, a higher DH was reached for Alcalase (19 %) than for Prolyve (15 %) after 5 hours of hydrolysis. This shows that the reaction cannot be standardized based on the activity determined by azocasein assay.
These results show that while the activity assay may be useful to guarantee reproducibility (if the same assay is used under the same conditions), it does not guarantee 'the same' activity for enzymes on different substrates. Hence, it is important to develop methods to provide an insight on the molecular mechanism of the enzyme action. This can only be done by characterizing the protein hydrolysates and in particular the kinetics of formation of single peptides

Influence of protein concentration

Performing enzymatic hydrolysis at high protein concentrations could result in a reduction of the amount of water and energy consumed during the production of hydrolysates. However, this benefit may be counterbalanced by a decreased efficiency of the process [6]. Indeed in this thesis, it has been shown that the overall hydrolysis rate as well as the DH reached after 2 hours was decreased with increasing substrate concentration, up to 30 % (w/v) WPI (chapters 2, 3 and 5). Besides a slower kinetics, increasing the protein concentration also results in the formation of gels. Before the formation of a gel, aggregates may be formed. The formation of aggregates has been studied at quite low concentrations of whey proteins during enzymatic hydrolysis by BLP (5 % (w/w)) [2] and hydrolysis of soy glycinin by chymotrypsin [7]. It has been shown that hydrolysis is hindered and eventually stopped by the formation of aggregates during hydrolysis [8]. In chapters 2 and 3, the formation of a gel during hydrolysis also hindered further hydrolysis and made it impossible to reach DH values higher than 15 % at the high protein concentrations. Because of the increase in viscosity and the formation of a gel, samples cannot be collected during hydrolysis. This hindered the complete description of the hydrolysis process at high protein concentrations. Such gelation has been previously shown to occur in a 20 % (w/v) WPI solution during hydrolysis by Alcalase [9]. Protease induced gelation has also been described for the enzyme BLP (*Bacillus licheniformis* protease) and has been mentioned to occur at concentrations as low as 2 % (w/v) α-lactalbumin [10] and for 12 % (w/v) WPI [11].
During data collection for chapter 2, the formation of viscous solutions leading to the formation of a gel was observed for hydrolysis of 30 % WPI by Alcalase (0.13 µL/mg protein) at initial pH 8.0 and 40 °C. Under these conditions, the hydrolysates form a gel after 2 hours of hydrolysis at a DH ±15 %. To confirm this visual observation an additional experiment is reported here. The viscosity of a 30 % (w/v) WPI solution during hydrolysis by Alcalase was monitored using a Rheometer Anton Paar MCR 501. Changes in complex viscosity were measured every 2 minutes for 6 hours. The temperature was kept constant at 40 °C during the analysis. The viscosity was constant in the first 5,000 seconds of hydrolysis. After that, the viscosity increased up to the

formation of a gel after 2 hours of incubation. During the viscosity measurement the pH was not controlled, which makes the conditions slightly different from the one used for the experiment in the pH-stat. (**figure 2**)

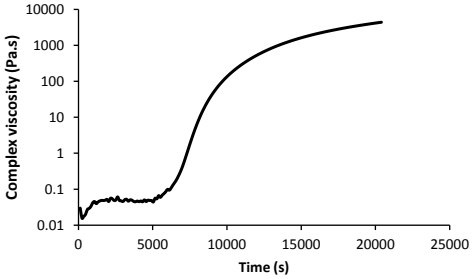

Figure 2. Viscosity of a 30 % (w/v) WPI solution during incubation with Alcalase.

It is important to mention that the viscosity is constant during the first 5,000 seconds of incubation while a slower rate of hydrolysis is observed from the first minutes of incubation onwards with increasing substrate concentration (chapter 2). This indicates that an increasing viscosity cannot be the reason for a lower rate of hydrolysis at elevated substrate concentrations. There may be formation of soluble aggregates, which might hinder the hydrolysis with increasing substrate concentration.
When 30 % (w/v) WPI is hydrolyzed by BLP at pH 8.0 and 40 °C, a gel is formed after the first minutes of the hydrolysis (chapter 3). This is the reason why the influence of substrate concentration could only be studied up to 10 % (w/v) for hydrolysis with BLP (chapters 3 and 5). (**table 2**) Alcalase is an a-specific enzyme, while BLP is a specific enzyme. That gels are obtained at two different time points or DH values during hydrolysis with the two enzymes is probably the result of the formation of different peptides.

Table 2. Influence of the hydrolysis conditions on the hydrolysates gelation at 30 % (w/v) WPI.

Enzyme	pH	Temp (°C)	Additives	Time of gelation
Alcalase	8.0	40		2 hours
BLP	8.0	40		5 min
BLP	8.0	40	1 % SDS	15 min
BLP	8.0	40	0.1 % LBG	50 min

SDS: sodium dodecyl sulfate; LBG: Locust bean gum.

The formation of a gel is hindering further hydrolysis and it also makes it difficult to handle the hydrolysates obtained. To try to avoid the formation of a gel and to be able to collect more samples during the hydrolysis by BLP of 30 % (w/v) WPI, a few additional trials were performed, which are reported here. This was done by addition of

additives to the solution before hydrolysis. (**table 2**) First, urea was added to a final concentration of 2 M or 4 M to a 30 % (w/v) WPI solution before hydrolysis. While this was done to avoid gelation, the addition of urea resulted in the formation of a gel at the initial conditions of hydrolysis (pH 8.0 and 40 °C). Secondly, SDS to a final concentration of 1 % (w/v) was added to the solution before hydrolysis. This resulted in a slight delay in the formation of the gel. A further increase of the concentration of SDS to 2 % (w/v) resulted in inactivation of the enzyme. Locust bean gum (LBG), a neutral polysaccharide, has been used in chapter 2 to determine the influence of viscosity on the hydrolysis rate for 1 % (w/v) WPI solution. The same polysaccharide was also added to a final concentration of 0.1 % (w/v) to 30 % (w/v) WPI for hydrolysis by BLP. The increase in viscosity of the initial solution actually led to a postponed formation of the gel, after 50 minutes of hydrolysis.

In conclusion, the formation of a gel during the hydrolysis of 30 % (w/v) WPI by BLP can be slightly postponed in time by addition of chemicals. Still, in all cases gels were formed at the higher concentrations, showing that this is a principle problem that cannot be easily solved.

In addition to the gel formation during hydrolysis, deviations in the properties of the initial protein solutions were observed with increasing protein concentrations. This has been shown by determining the conductivity of protein solutions as a function of protein concentration. Above 10 % (w/v) WPI the correlation between conductivity and protein concentration deviates from linearity (chapter 2). Another deviation from linearity has been observed by measuring the fraction of free water by relaxation time using NMR. For concentrations of 20 and 30 % (w/v) WPI a different linear regime was observed than for protein concentrations of 0.1 to 10 % (w/v) WPI (chapter 3). This indicates a deviation from linearity, i.e. ideal behavior. Ideal systems are defined as systems in which the number of molecules is so low that they do not influence each other [12]. By increasing protein concentration, as seen by the conductivity, concentration dependent effects cannot be neglected. Such solutions are called non-ideal. One reported observation is the increase in protein stability in crowded systems. Molecular crowding is commonly used to describe the effect of increased stability of molecules in the interior of cells, where the total concentration of macromolecules is high (i.e. up to 400 g/L) [12]. This is experimentally studied by addition of a high concentration of a crowder to a solution of protein. Molecular crowding effects are also sometimes called excluded volume effects. It is generally assumed that the hydration structure of proteins is altered in the presence of crowder molecules, which are either small molecules (e.g. glycerol) or macromolecules (e.g. PEG) [13]. Based on the non-ideal behavior of conductivity and decreased hydrolysis, in combination with the theories of molecular crowding, it can be assumed that the hydration at concentrations as high as 30 % (w/v) WPI deviates from the hydration of proteins in a diluted (ideal) solution.

The observed gelling behavior of concentrated systems (at 30 % (w/v) WPI) seems to be a fundamental property of such concentrated systems and could not be suppressed or avoided by adding SDS or urea. For samples at lower concentrations, no gel was

formed, while still a deviation from ideality was noted. In conclusion, these examples show the difficulty in understanding and describing behavior of highly concentrated protein solutions due to their non-ideality.

Inactivation of the samples taken during hydrolysis

For a correct characterization of the hydrolysates, changes in the peptide profile due to storage or enzyme inactivation should be avoided. A mild method would be to separate the enzyme from the hydrolysate by the use of a membrane [14]. When the enzyme is separated using a membrane, the enzyme is not inactivated and can potentially be re-used. However, at low DH for instance, high molecular weight peptides and remaining intact proteins will not be separated from the enzyme. Consequently, not all peptides are recovered in the sample. With this technique, the samples obtained cannot be used for detailed analysis of the peptides. An alternative would be to immobilize the enzymes on e.g. resin beads. This would allow the remaining intact proteins to be separated from the enzyme [15]. With this, the hydrolysates are not altered but the cost of production of hydrolysates is increased. For research applications, typically enzymes are inhibited using protease inhibitors, heat inactivation or pH inactivation.

The use of protease inhibitors can result in enzyme inactivation without further changes (i.e. due to heating) in the hydrolysates. However, different enzymes will require the use of different inhibitors. Moreover, for some proteases no efficient inhibitors are known or available. For instance, for BLP six known serine protease inhibitors have been tested but none of the inhibitors were able to inhibit the enzyme [16]. Both irreversible and reversible inhibitors have been described. Irreversible inhibitors can bind covalently and permanently to the active site of the substrate, mimicking the tetrahedral intermediate formed during hydrolysis [17]. In some cases, the binding leads to a distortion of the active site of the enzyme [17]. It is known that the efficiency of protease inhibitors depends on the conditions under which they are added to the enzyme. In conclusion, the use of protease inhibitors has the advantage that it does not alter the hydrolysate mixtures. On the other hand, for industrial processes compared to other inactivation techniques, the use of inhibitors increases the cost of hydrolysates production. Also, as mentioned above, for some proteases no effective inhibitors are known. This was the case for the BLP used in our studies.

A third method frequently used to stop enzymatic reactions is heat treatment. To inactivate the enzyme, the hydrolysates are typically heated for 10 to 20 minutes at temperatures varying from 80 °C for trypsin, pancreatin and pepsin [18], 85 °C for chymosin [19], 90 °C for Alcalase [5] and up to 95 °C for Flavourzyme [20]. None of these studies mention the possible influence of heat denaturation on the solubility, especially at low degrees of hydrolysis, and on the molecular weight distribution of the peptides obtained. It has, however, been shown that heat treatment of hydrolysates does affect the composition of the hydrolysates as well as their bio-functional properties

[21]. For instance, the scavenging activity of the hydrolysates towards DPPH and O_2 was reduced (by 45 and 10 %, respectively) after heat treatment [21]. This can be due to changes in composition or degradation of certain amino acids. Heat treatment of 30 and 15 min, at 60 °C and 120 °C, respectively, has been reported to degrade the high molecular mass peptides fraction (> 5 kDa) into peptides with lower molecular mass or free amino acids at neutral pH [21,22]. Still at neutral pH cleavages of peptide bonds are not expected even at high temperature. Moreover, heat-treatment can cause aggregation at neutral pH [23], or changes in the bioactivity of the hydrolysates [21]. These effects have been demonstrated for temperatures which are used for enzyme inactivation (< 100 °C). In an industrial setting, the hydrolysates will probably anyway be pasteurized. Hence, it is expected that heat treatment leads to alteration of the peptide profile or to aggregation, which hinders the detailed scientific study of the mechanism of hydrolysis.

Finally, the last alternative to inactivate the enzyme present in hydrolysates is pH inactivation. This is done by changing the pH of the solutions for only a few minutes to extreme pH values, such as pH 2 for BLP [16] or pH 11 for pepsin [24]. It should be noted that only a small number of enzymes are irreversibly inactivated by pH changes. Trypsin, for instance, is inactivated at pH 11, but regains its activity when the pH is re-adjusted to pH 7-8 [25]. The changes in pH for a limited time have a negligible effect on the peptide stability. However, changes of pH have been described to have an effect on the solubility of the hydrolysates [26].
In this thesis, Alcalase and BLP were inactivated by adjusting the pH to 2. As described in chapter 2, changing the pH to 2, irreversibly inactivates Alcalase. This has been shown for BLP is in a previous study [16]. The effect of pH inactivation on the solubility and stability of the hydrolysates was studied as preliminary experiment before detailed analysis of the hydrolysates. To reach pH 2.0, HCl was added to the hydrolysates and the sample was neutralized again for storage (chapters 4, 5 and 6).
The solubility was measured by determining the protein content in the supernatant after centrifugation and compared to the total protein content. The solubility tested before pH treatment showed that the hydrolysates had a solubility of on average 95 %. The solubility was also high (95 %) at pH 2.0 but the step from pH 2.0 to pH 8.0 is the step leading to a decrease in solubility. As a result, the proportion of soluble peptides was decreased with the increase in DH, down to a soluble proportion of 40 % at DH 4 % after adjusting the pH back to pH 8.
To solve the problem of insolubility, the samples were stored at pH 2.0 before further analysis. To test the effect of storage, the DH was determined on the hydrolysates using the OPA assay. Higher DH were measured by OPA than what was expected based on the pH-stat method. At the same time, to confirm the results observed with the OPA assay, one sample was analyzed by RP-UHPLC after storage at pH 2.0 after two and four months. (**figure 3**)

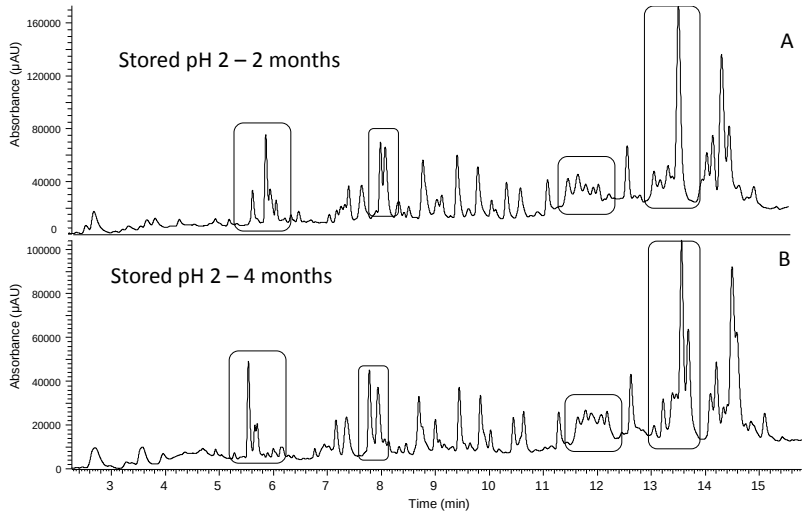

Figure 3. RP-UHPLC chromatograms of one hydrolysate (5 % WPI - DH 4.5%) stored at pH 2.0 and at -20 °C (A) for 2 months and (B) for 4 months.

Large differences in the chromatograms were observed, which were attributed to acidic hydrolysis taking place in the sample stored at pH 2.0. Therefore, the samples should be stored at pH 8.0. The DH measured by OPA assay on hydrolysates stored at pH 8.0 were similar to the DH values obtained by the pH-stat method, indicating no degradation of peptides. This was confirmed by injecting samples stored at pH 8.0 on RP-UHPLC-MS. (**figure 4**)

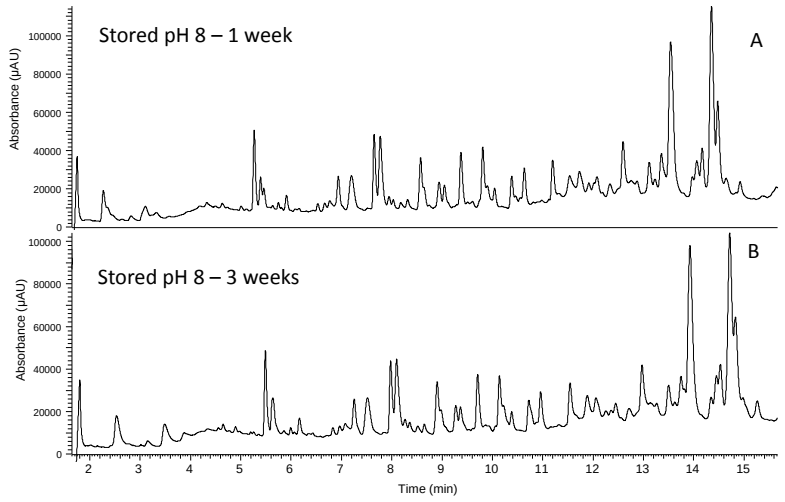

Figure 4. RP-UHPLC chromatograms of one hydrolysate (5 % WPI - DH 4.5%) stored at pH 8.0 and -20 °C (A) for 1 week and (B) for 3 weeks.

No changes in the chromatograms were observed. At pH 8.0, the peptides aggregate after the acidic inactivation of the enzyme, but sample preparation and injection into

LC-MS is performed at acidic pH. It was found that under these conditions the peptides are soluble again. In conclusion, making use of the reversibility of the aggregation, the samples can be stored at neutral pH and analyzed at acidic pH without losses resulting from aggregation. Once the enzyme is properly inactivated, the hydrolysates can be characterized in detail.

Challenges in peptides annotation and quantification

If the bio-activity of hydrolysates is studied, typically only a few specific peptides are identified since these are considered the most important ones with respect to the studied functionality (for instance VPP and IPP for ACE inhibition [27]). In the same way, in proteomic studies the tryptic digests of cells or tissues are used to identify which proteins are present. For the identification of such proteins, a limited number of peptides (i.e. 1 to 10) is considered to be sufficient. Since these studies are not aimed at a full complete annotation of the hydrolysate obtained, there were no established methods to annotate all peptides in an hydrolysate at the start of our research. Such methods are essential to fully characterize the enzyme activity.

In this thesis, several steps were identified that are important for a full and complete description of peptides in hydrolysates. The first point for complete analysis is a good sample preparation in which losses, due to insolubility, are avoided. Secondly, the annotation of all peptides present as well as their quantification is the key for complete description. Finally methods to validate the analysis (i.e. annotation and quantification) are also necessary.

Sample preparation

Sample preparation is a crucial step in any analytical method. Typically, samples are diluted (or redissolved) in the eluents used for analysis. However, hydrolysates contain a large variety of molecules (from intact proteins to free amino acids). Consequently, it may be that in a particular solvent, part of the sample will not be completely soluble. While losses on the column can be avoided by solubilizing the samples in the initial conditions of elution, it may mean that not all peptides are dissolved and analyzed.

Another issue is the presence of disulfide bridges between peptides. In chapters 4, 5 and 6, the samples were incubated in DTT to reduce the disulfide bridges, to simplify the annotation of the peptides. This will increase the completeness of identification and quantification. At the same time, the obtained information cannot be directly used to understand the functionality of the hydrolysate, since this will be affected by the presence of the disulfide bridges in the peptides.

Only if all peptides are injected in LC-MS, complete annotation of the parental protein sequence can be achieved. This is not commonly verified in studies on protein hydrolysates. In this thesis, two parameters have been taken into account to verify that all peptides were indeed injected. First, the solubility of the samples was tested to ensure that no losses occurred during enzyme inactivation. In addition, the total UV

area, being the sum of UV response of the peptides and remaining intact proteins present, of all samples was compared. In all experiments the total UV_{214} signal decreases with increasing DH. The losses are generally higher than expected. A part of the losses is the result of a decrease in the number of peptide bonds. In detail, about 50 % of the value at 214 nm for proteins (e.g. 293406 $M^{-1} \cdot cm^{-1}$ for β-lactoglobulin B) are contributions from the peptide bonds [28]. Consequently, at DH 7 % for instance, the contribution of the peptide bonds is decreased by 3.5 %. The unexpected losses might be due to the presence of free amino acids and dipeptides which elute at the beginning of the run and thus are not recovered in the UV chromatogram. Once the sample preparation is validated, detailed analysis can be performed.

Peptide Annotation

At the start of this research project, the chromatograms were entirely manually analyzed, which is time-consuming. To reduce the amount of time spent per chromatogram, the development of a fast and reliable, automated annotation method was necessary. This was done using the software Biopharmalynx 1.3 (Waters). Any automated method will be a compromise between the selectivity (only select signals that are strong and correct) and the completeness (including low signals). However, the inclusion of low intensity signals unavoidably leads to an increased error in the assignment. The aim is to have as many correctly annotated peptides as possible, with a minimum of incorrectly assigned peptides. Preliminary set-ups of the method were tested on a chromatogram that was first manually annotated. To optimize the method, the limit of detection of the MS relative intensity and the mass tolerance were varied. The intensity limit of detection was finally set to 10 counts and the mass tolerance to 0.1 Da (chapter 4). Then, the annotation of b and y fragments was included to ensure the quality of the identification. The b and y fragments are formed during MS analysis by increasing the energy in the MS system. They are the result of the cleavage of peptides on peptide bonds, from the N-terminal and from the C-terminal end, respectively. The limit of detection and the mass tolerance of MS/MS peaks were also optimized to include b and y fragments. These parameters were set to 10 counts and 0.1 Da for the MS/MS peaks, respectively. In the final automated method, a number of differences were found with the manual annotation. Some peptides were incorrectly annotated, while others were not detected within the set limits. Several reasons can explain these differences from manual to automated annotation.

1. In-source fragmentation. Fragments obtained by in-source fragmentation can be identified as fragments because they have the exact same elution time as the parental peptide. In this way they can be easily removed manually. The software used here, however, does not distinguish peptides and fragments that result from in-source fragmentation present in the MS chromatogram. This leads to annotation of some fragments as if they were peptides. The problems with automatic identification of fragments resulting from in-source fragmentation has been discussed for tryptic

hydrolysates using a different software [29]. In that case the software assigned most of the y fragments to a-specific tryptic peptides (i.e. with an a-specific cleavage according to the enzyme specificity, occurring on one side or both sides of the peptide) [29]. Hence, manual correction is needed to differentiate the fragments from the peptides.

2. Multiple charge states. Long peptides with high molecular masses (above 3 kDa) are ionized to a higher multiple charge state than short peptides. They are found in the MS with high multiple charge states (3+, 4+) or even up to 11+ for intact β-lactoglobulin. These peptides, as well as the intact protein are generally not detected or interpreted correctly by the software. This is probably due to the fact that the distance between isotopes is decreasing by increasing the charge state. This makes it difficult to assign the correct charge state and consequently to identify a peptide. In addition, the difficulty to assign these peaks automatically by the software is increased if the intensity is low, since baseline noise further hinders correct identification. The annotation was corrected manually.

3. Isobaric peptides. Even in our relatively simple model system (WPI), several peptides, with similar molecular masses were formed. For instance, peptides β-lg(A)[115-127] and α-lac[12-25] have a mass of 1474.7 and 1474.8 Da, respectively. The automated method used in this thesis primarily looks at the mass and confirms the annotation with b and y fragments. The method does not use the combination of mass and fragments to assign the correct peptides, or it does not look for a second match if the annotation is not confirmed by the fragments. This has frequently led to wrong annotation of these two peptides with similar m/z by the software. This can be solved manually by determining the exact molecular mass and assigning the b and y fragments. For a search on peptides obtained within the enzyme specificity with only two or three proteins, this remains an exception. In more complex system, with either more proteins or when an a-specific enzyme is used, the problem will be more frequent.

In conclusion, the automated method is proven to be valuable, but still a number of manual identifications and corrections are needed. A final addition in the automated method, which may increase its applicability, is to link the annotated peptides to the corresponding UV peak areas. The current software only gives an output file of the peptides based on the retention time. For quantification purposes, the peptides identified have to be assigned manually to the corresponding UV peak areas.

Quality of peptide annotation

The completeness of the identification is commonly described by calculating the amino acid sequence coverage, both in proteomics studies [30] and for enzymatic hydrolysis studies [31,32]. As discussed in chapter 4, this is only a poor indication of the completeness, since this parameter does not take into account the fact that the same amino acids can be annotated in several peptides. For this reason, in this thesis the peptide sequence coverage has been defined as a better evaluation of the number of

annotated amino acids (chapter 4). The peptide sequence coverage is defined as the number of amino acids in annotated peptides divided by the total number of amino acids present in all (annotated and missing) peptides. In the different samples annotated at increasing DH, it was observed several times that with an amino acid sequence coverage of 100 %, the peptide sequence coverage was 90 %. This was for instance the case for 1 % (w/v) WPI at DH 6 and 7 %. Such a peptide sequence coverage indicates a quite complete annotation, but still that not all peptides that should be present were annotated. In these examples 37 peptides were annotated and based on these peptides annotated, it is determined that 7 were missing.
The missing peptides might be short peptides or even free amino acids that are not easily annotated in RP-UHPLC-MS. Hydrophilic peptides elute at the start of the run and are consequently not present in the chromatograms. In addition, the small masses of dipeptides or free amino acids can easily be missed in a mass spectrum, because the masses are close to the noise of the MS signal.
It was concluded that peptide sequence coverage should be the reference to evaluate the quality of the annotation. This value gives a more complete description of the hydrolysates than the commonly used amino acid sequence coverage.

Peptide quantification

After annotation and after verifying the quality of the annotation, peptides can be quantified. In this thesis, the quantification of peptides was based on the UV_{214} peak area. This approach was introduced in 2007 in our group [8]. The molar-based quantification technique in LC system was further developed in 2012 [33] and subsequently used (e.g. [32,34,35]). For complete description, in this study, peptides were annotated and quantified up to 90-95 % of the UV_{214} of each chromatogram. This type of consideration, (percentage of UV signal explained) is not commonly done, because quantification of all peptides present in an hydrolysate is not common. Proteomics studies base their quantification on the MS signal and thus describe the percentage of MS data analyzed. In one study, for instance, the extent of the identification was described to be two thirds of MS/MS spectra [36]. This gives no indication on the actual amounts of peptides annotated.
The quality of the quantification is determined by quantifying each amino acid from the parental protein sequence based on the quantification of the peptides. Each individual amino acid in the protein sequence should be present at the same molar concentration as the initial protein concentration before hydrolysis. The completeness of the quantification is visually determined by the plot of the concentration of each amino acid of the protein sequence. (**figure 5**) The difficulty is to be able to describe the quality by a unique parameter. To describe the quality, a first parameter was developed, referred to as molar sequence coverage(R^2) (%). This was calculated as the coefficient of determination R^2.

(1) $$Molar\ sequence\ coverage_{R^2} = 1 - \frac{\sum_{n=1}^{162}(C_n - C_o)^2}{\sum_{n=1}^{162}(C_n - \bar{C})^2} \times 100$$

Where C_n (μM) is the concentration of each amino acid n in the protein obtained from quantification of the peptides; C_0 is the initial concentration of the protein (μM) (which is also the concentration of each amino acid in the sequence) and $\bar{C}$ is the average concentration (μM) of C_n calculated by $\bar{C} = \frac{\sum C_n}{n}$. This value gives an indication on how good the recovery is for each amino acid in the parental protein sequence based on the theoretical protein concentration and on the average concentration. The molar sequence coverage(R^2) was on average 75 % for β-lactoglobulin (chapters 4, 5 and 6), which is considered as a quite complete annotation. Still for some samples, the value does not reflect exactly the quality of the quantification. For instance, if all amino acids are under-estimated, with a good average, 90 % of the expected concentration, the R^2 determined will be low (e.g. 50 %). If the data points are not distributed below and above the expected value, the molar sequence coverage(R^2) gives an incorrect representation of the quality. Because of some misleading values of the molar sequence coverage(R^2) in some cases, (i.e. no distribution of the points below and above the expected concentration), a second calculation was developed and used in the different studies presented in this thesis. The molar sequence coverage was expressed in this second calculation as (1 - σ) in which σ is the standard error as described by equation 2 (chapters 4, 5 and 6).

(2) $$Molar\ sequence\ coverage = \left(1 - \frac{\sqrt{\frac{\sum(C_n - C_0)^2}{(\#AA-1)}}}{C_0}\right) \times 100$$

Where C_n and C_0 are the concentrations (μM) of each amino acid n in the protein and the initial concentration of the protein, respectively. #AA is the number of amino acid residues in the sequence. Using this value a more complete description of the quality of the quantification was obtained.

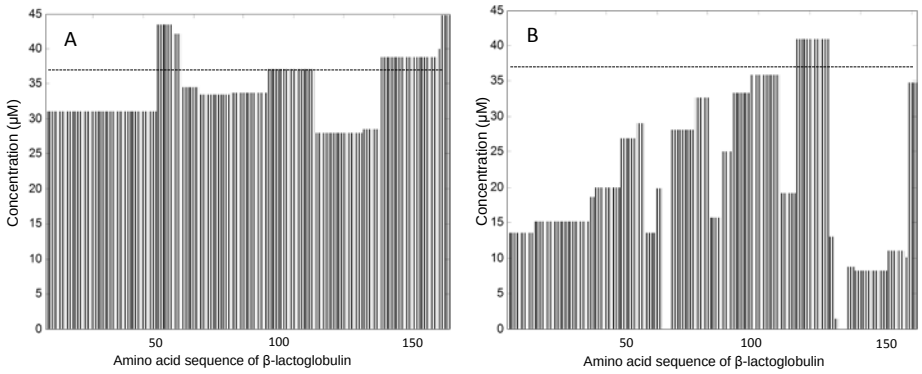

Figure 5. Concentration of all amino acid residues in β-lactoglobulin during hydrolysis by BLP of (A) 1 % WPI at DH 1.5 % (R^2 = 42 % and molar sequence coverage = 85 %) and of (B) 10 % WPI at DH 6% (R^2 = 70 % and molar sequence coverage = 50 %).

Quality of the annotation and quantification as a function of the DH

At a low degree of hydrolysis (DH = 1.5 %), 25 peptides between 1 and 7 kDa were on average annotated for all experiments. Simultaneously, 100 % amino acid sequence coverage is reached and a peptide sequence coverage of 98 % is annotated. The molar sequence coverage as indication of the quantification is high at low DH around 80 %. With increasing DH up to DH 7 % in this study, an increasing number of peptides is formed, with shorter sequences or even single amino acids. With further increase of the hydrolysis the number of peptides will be decreasing. Only 84 peptides resulting from hydrolysis of β-lactoglobulin (A + B) were annotated over all analysis while 658 peptides are expected in total for the hydrolysis of β-lactoglobulin (A + B) with BLP, as explained later in the text. As mentioned in chapter 8 some peptides might be missed due to their low concentrations. From the modeling of a theoretical protein with cleavage sites at regular intervals it was indeed shown that the concentrations of long peptides containing high number of cleavage sites is low. Still as a result of the increasing number of peptides, the overall UV signal is spread over a large number of peptides. Furthermore, the total UV signal is decreased compared to the low DH because less peptide bonds are present. At high DH values, the amino acid sequence coverage is not always 100 % and the peptide sequence coverage is also lower than at low DH, down to 90 %. The quantification as determined by the molar sequence coverage is also decreasing at high DH, down to an average of 65 %. (**figure 5**) In addition, because more peptides are present, there are also more peptides co-eluting. In case of co-elution, the UV signal was divided over the co-eluting peptides based on MS intensity. This is, as explained in chapter 4, based on the assumption that peptides co-eluting should have comparable ionization. Finally, some inaccuracies in the quantification might result from inconsistencies in the automatic integration by the software. Depending on the time range selected for integration, the baseline determined by the software will be slightly changed leading to slightly different UV peak areas.

At high DH, on two different parts of the sequence of β-lactoglobulin (between positions 63 and 66 and between 129 and 131) no signal is quantified, probably as the result of the formation of dipeptides or single amino acids that are not annotated. In these parts of the sequence there are indeed several possible cleavages sites next to each other. (**figure 5**) Five free amino acids can theoretically be formed during incubation of β-lactoglobulin with BLP. The resulting free amino acids are 45-E, 130-D, 131-E and 158-E but also 65-E in β-lactoglobulin (A). Free amino acids are not easily detected in MS due to their low molecular weight. In addition, on a reversed phase UHPLC column, free amino acids and dipeptides containing glutamic acid are most likely hydrophilic and eluting at the beginning of the gradient [37]. In addition, the free glutamic acids have a low UV_{214} signal [28] and are consequently difficult to quantify.

For two parts of the parental protein sequence β-lg[1-45] or β-lg[135-157], only 30 % of the expected signal is annotated at high DH. On one hand the total UV signal is decreasing as a function of DH. On the other hand, 90 % of the total UV signal is

annotated even at high DH. This indicates that the missing signal might not be related to the lack of annotation, but rather to losses during sample preparation or elution. Peptides β-lg[1-45] and β-lg[135-157], for which an incomplete quantification is obtained at high DH, are known to be involved in peptide-peptide interactions leading to aggregation and insolubility [34,38,39].
The developments of these quality parameters enable the description of the completeness of the peptide analysis. Only in this way certainty can be obtained on how much of the sample has actually been annotated and quantified. In addition, based on the values obtained by these parameters, the final analysis was improved by subsequent manual analysis of the samples.

Enzyme specificity

Enzymes are usually defined by their specificity, which indicates after which type of amino acid the enzyme will cleave. The enzyme BLP, used in this study for peptide analysis is considered to be a pure enzyme preparation with 78 % of BLP and 14 % of pro-peptide as determined based on the UV_{214} peak area (chapter 3). The enzyme has a specificity for two types of amino acid residues (i.e. Glu and Asp) [40]. Still, in the different peptide analyses (chapters 5 and 6) a number of a-specific peptides have been annotated. The number of these a-specific cleavages and their concentration is correlated to increasing incubation time. In addition the a-specific cleavages were found to occur after several types of amino acids. In the past, such a-specific peptides have often been attributed to side-activities (impurities) present in the enzyme preparation. As described in chapter 7, it was found that the a-specific cleavages annotated were the result of spontaneous cleavages of specific peptides with specific sequences. This can explain many unexpected cleavages in the study of β-lactoglobulin hydrolyzed by BLP. It was concluded that the secondary structure of the peptide, which depends on the primary sequence, is the reason for additional strain on specific bonds. This strain results in breakage of the bonds. The cleavage of the bond is enhanced in the presence of the enzyme. Based on these conclusions, it is suggested that in future studies the formation of unexpected peptides should be reconsidered. A-specific cleavages are typically assumed to be the result of side activity or impurities in the enzyme preparation, but it was shown that they are rather peptide dependent. The phenomenon should not be neglected considering that it was confirmed for 4 peptides in the hydrolysate of β-lactoglobulin alone.

Determination of the selectivity

Mechanism of hydrolysis

After complete peptide identification and quantification, the mechanism of hydrolysis can be studied. To determine the mechanism of hydrolysis, there were no established methods at the start of this study. Describing the mechanism of hydrolysis implies that all data obtained in terms of peptide identification and quantification can be expressed

by a unique parameter or a limited number of parameters. To describe the sequence of events in enzymatic protein hydrolysis, we introduced the selectivity as a quantitative parameter. The selectivity determined in this study is defined as the relative rate of hydrolysis for a cleavage site compared to the sum of rate of hydrolysis of all cleavage sites in the parental protein (chapter 4).

To calculate the selectivity, first the apparent cleavage rate is determined. The rate of selective hydrolysis is derived from the apparent cleavage rate (chapter 4). The apparent cleavage rate for each cleavage site is determined by fitting the concentration of cleavage products as a function of time using a first order kinetic equation. For most of the cleavage sites (12 out of 17 on average) this is an adequate fit of the data. In some cases, specifically for cleavage sites that are hydrolyzed at later stages of the hydrolysis, the fit is not good. However, alternative fitting methods will only introduce more fitting parameters. The increase in fitting parameters results in increasing uncertainty on the exact value of the parameters. In conclusion, the first order kinetics was the most efficient fit for all cleavage sites resulting in a single parameter to describe the rate of hydrolysis of each cleavage site.
Selectivity was successfully developed as a parameter to describe quantitatively the events during enzymatic protein hydrolysis. Based on this newly developed parameter one of the major conclusions of this thesis was established: it was concluded quantitatively that not all cleavage sites in a protein are hydrolyzed equally by the enzyme (chapter 4). The reasons for these differences in the rate of hydrolysis are not really clear. The explanations for this observation can most likely be found in the primary structure of the parental protein.

Factors influencing the hydrolysis

With selectivity as a new quantitative parameter, the hydrolysis process can be described and the influence of system conditions, such as the increase in protein concentration or the changes in pH, can be quantitatively studied.
Increasing substrate concentration during enzymatic protein hydrolysis results in lower hydrolysis and lower DH reached (chapter 2). The determination of the selectivity towards all bonds in β-lactoglobulin during hydrolysis of 0.1 to 10 % (w/v) WPI also showed changes in the mechanism of hydrolysis (chapter 5). For the four cleavage sites in β-lactoglobulin with the highest selectivity a clear influence of the substrate concentration is observed. For two cleavage sites a decrease by a factor 2 is observed at 10 % (w/v) WPI compared to 1 % (w/v) while for the other two an increase by a factor 2 is observed. In addition, some effects of substrate concentration are observed for other cleavage sites with lower selectivity but these cleavage sites have consequently less influence on the overall composition of the hydrolysate. For instance, the selectivity towards aspartic acid residues is increased with increasing substrate concentrations, but the selectivity towards these cleavage sites is on average 1000 times lower than for the cleavage sites after glutamic acid residues. The method developed was

successfully applied to hydrolysates at different initial concentrations. Based on this first use of the method, it is assumed that the method can be further optimized and applied to determine the mechanism of hydrolysis in view of a control of enzymatic hydrolysis toward specific peptides.

The influence of the pH on the hydrolysis kinetics and on the selectivity of the enzyme was also determined. The susceptibility for hydrolysis of intact β-lactoglobulin is increased with increasing pH from 7.0 to 9.0. This correlates with a decreased stability of β-lactoglobulin at increasing pH (chapter 6). This showed the influence of the protein structure and stability on the rate of hydrolysis. The mechanism of hydrolysis was compared by determining the selectivity at three pH of hydrolysis. For these conditions, it is confirmed that not all cleavage sites are hydrolyzed equally by the enzyme. In addition, significant changes were observed for the selectivity of certain cleavage sites as a function of pH with either large increase or large decrease. Because the changes do not follow a unique trend as a function of the pH of hydrolysis, no clear explanation was found based on the primary structure of the protein.

Correlation between simulation and experimental data

A stochastic model was developed to describe the mechanism of enzymatic protein hydrolysis (chapter 8). The model was first tested on theoretical peptides. With this, it was established that the position of the amino acid in the sequence as well as the number of cleavage sites in the peptide changes the probability for the peptide to be hydrolyzed. In addition, while the enzyme affinity is set to be the same for all cleavage sites, different selectivities towards all cleavage sites were obtained. This confirmed the conclusion of chapter 4 that the cleavage sites after the same type of amino acid do not have the same selectivity.

Secondly, it was found that by changing the chance to hydrolyze the intact protein compared to the peptides (i.e. accessibility of the protein), changes in the correlation between remaining intact protein and DH are observed. This observation correlates with the different behavior of remaining intact protein obtained experimentally for different pH of hydrolysis (chapter 6). This confirmed one of the conclusions of chapter 6 that the stability of the protein has an influence on the mechanism of hydrolysis.

Based on this, the changes in protein accessibility in the model were used to mimic the behavior observed at different pH values of hydrolysis to compare the enzyme selectivity. The affinity was at first set to the same value towards all cleavage sites. The data obtained from the simulation showed the same types of behavior as the experimental data as a function of the pH (chapters 8 and 6). For some cleavage sites an increase or decrease as a function of protein accessibility are observed while for others an optimum selectivity is observed. This indicates that by setting the same affinity towards all cleavage sites, different values of the selectivity are obtained. Some improvements of the prediction of the selectivity were obtained by changing some parameters of the simulation. This included setting a lower affinity to cleavage sites for which an experimental selectivity was found to be lower or by including the aspartic

acid residues in the simulation. With these parameters the selectivity predicted was improved for some of the cleavage sites, but deteriorated for others. This showed that the selectivity for a certain cleavage site is influenced by the neighboring cleavage sites and by the affinity towards the neighboring cleavage sites.
In conclusion, the use of such model and approach was a separate additional validation of the quality and interpretation of the experimental data. This also showed that the experimental data or part of the data can be explained with a statistical approach of the enzyme action. The fact that not all data can be explained shows that it is difficult to account for all the parameters influencing the selectivity (chapter 8).

Extrapolation to complex systems

The method of annotation and quantification was developed for a simple system taking into consideration two proteins, β-lactoglobulin and α-lactalbumin using a specific enzyme (chapter 4). In addition, variants A and B of β-lactoglobulin were both present. For pure β-lactoglobulin A 406 peptides are formed. β-lactoglobulin B contains one cleavage site less than β-lactoglobulin A so a total of 378 peptides are formed from variant B. If both proteins are present, 126 similar peptides will be formed and in addition, 280 peptides unique for variant A and 252 for variant B resulting in a total of 658 peptides. Experimentally, 11 peptides resulting from variant B were annotated and 13 for variant A. By using a specific enzyme, the peptide annotation is relatively simple compared to a-specific enzymes. Hence, the methods developed in the course of this PhD thesis were tested on a simple model but have a potential for a use in more complex systems.

Increasing the number of substrates

In the WPI used, β-lactoglobulin is present in a higher proportion than α-lactalbumin. By determining the proportion of remaining intact protein as a function of the DH determined by the pH-stat, it is apparent that β-lactoglobulin is hydrolyzed preferentially compared to α-lactalbumin. (**figure 6**)

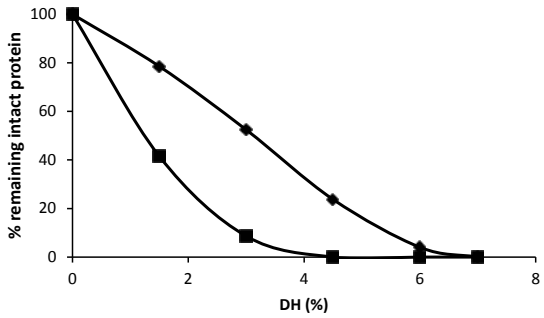

Figure 6. Proportion of intact (♦) α-lactalbumin and (■) β-lactoglobulin as a function of DH determined by the pH-stat method, for 5 % WPI hydrolyzed by BLP.

This is simply explained by the fact that β-lactoglobulin is present in a higher amount than α-lactalbumin and that there are relatively more Glu residues in β-lactoglobulin than in α-lactalbumin. The experimental observation was successfully modeled by taking into consideration a number of molecules for both proteins relative to the WPI used experimentally (chapter 8). After DH 4.5 %, a number of peptides (± 10 peptides), resulting from the hydrolysis of α-lactalbumin were annotated with good peptide sequence coverage (98 %) and an acceptable molar sequence coverage of 65 %. (chapter 4) The presence of two proteins already increases the chance of mis-annotation based on MS data as described in the previous section. The peptides β-lg(A)[115-127] and α-lac[12-25], for instance, have a comparable molecular mass, 1474.72 and 1474.80 $g.mol^{-1}$, respectively. This can lead to confusion if the m/z is not accurate enough or the system poorly calibrated or if the settings are too restrictive on the m/z. The correct annotation can be done by assigning b and y fragments as mentioned previously. Peptides with similar molecular mass will be more frequently present by increasing the number of parental proteins. For future analysis and description of the hydrolysis mechanism of more complex systems than WPI, the hydrolysis of soy protein isolate might be considered. Soy proteins hydrolysis occurs in several industrial processes. Soy proteins isolate is a mixture containing glycinin and β-conglycinin. For glycinin, 6 different subunits have been identified and each glycinin subunit contains an acidic (± 32 kDa) and a basic polypeptide chain (± 20 kDa). β-Conglycinin consists of 3 subunits (± 65 kDa). Another example of complex analysis can be obtained after hydrolysis of patatin (41 kDa), which is not a unique protein but a mixture of 7 isoforms. The complexity is also increased due to the higher molecular masses of these proteins compared to β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa).

Using a-specific enzymes

A step further in understanding digestion of protein is to describe the hydrolysis of a protein or a mixture of proteins hydrolyzed by an a-specific enzyme. As a preliminary experiment we report here on the annotation and quantification of the peptides present in an hydrolysate (DH = 2 %) obtained by hydrolysis of 1 % WPI by Alcalase. The hydrolysis was performed in the conditions used in chapter 2. The peptide annotation was performed using the automated method developed for BLP hydrolysates of WPI. In total 80 peptides were annotated by the software. These peptides resulted from the hydrolysis of β-lactoglobulin and α-lactalbumin according to the software. Fifteen annotated masses were incorrectly interpreted by the software as determined by subsequent manual annotation. By checking the annotation manually, it was observed that for each m/z there are in general several possible peptides using a tolerance of ± 0.5 Da on the final molecular mass of the peptide. In most of the cases, the annotation of the b and y fragments is required to identify the correct peptide. Since the software does not consider simultaneously the molecular mass and the b and y fragments, isobaric peptides are often not correctly assigned. Only by including these two

parameters (closest mass and number of fragments identified), correct annotation can be achieved. The software itself should be improved to obtain better results. After manual correction, 90 % of the UV_{214} peak area was assigned to peptides resulting from β-lactoglobulin and α-lactalbumin and to intact remaining proteins. This resulted in an amino sequence coverage of 100 % and a peptide sequence coverage of 91 % (**figure 7**), synonym of a good quality in the annotation, comparable to what was obtained for a specific enzyme. Cleavages were annotated after 16 amino acid residues, principally after Leu (22), Glu (17) and Asp (7) residues, but also after the aromatic amino acids (Phe (10), Trp (3) and Tyr (5)).

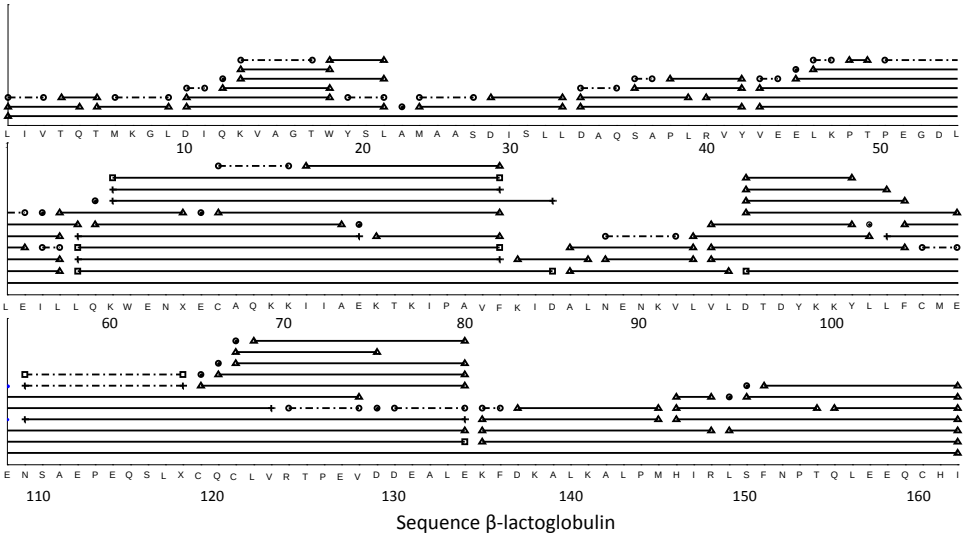

Figure 7. Peptide sequence coverage during hydrolysis of 1 % WPI at DH 2 % hydrolyzed by Alcalase. The full lines (△——△) represent the annotated peptides and the dotted (○– – –○) lines represent the missing peptides. △ are used for peptides from both variants, + and □ are used for peptides annotated for variant A and B of β-lactoglobulin, respectively. The X in the amino acid sequence refer to amino acid 64 and 118 which differ between β-lactoglobulin variant A and B.

The molar sequence coverage was 60 %, lower than the average obtained for the specific enzyme BLP. (**figure 8**) This value of molar sequence coverage indicates that on some part of the sequence, the expected concentration of certain amino acids was not recovered. This can be due to a loss of peptides in the sample preparation or due to wrongly assigned masses.

The underestimation might also be the result of the high number of peptides co-eluting, which might not always be correctly identified. A better separation might improve the annotation and quantification but co-elution will remain present in such a mixture with so many peptides. By improving the separation, the quantification might be improved. The underestimation might also be the result of the formation of free amino acids, as

explained previously. In addition, a different MS system with increased sensitivity could also help in improving the annotation of peptides with low ionization.

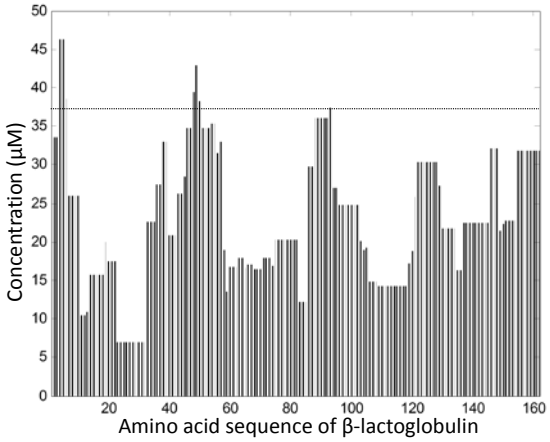

Figure 8. Concentration of all amino acid residues in β-lactoglobulin for hydrolysis of 1 % (w/v) WPI by Alcalase at DH = 2 %

Finally, based on this preliminary interpretation of a more complex hydrolysate, it is concluded that the tools developed for a simple model during this PhD project can be used for complex systems as well. The software, however, would have to be improved to use the different criteria (mass tolerance and identification of fragments) at the same time rather than successively. The other option would be that the annotation is rejected if not enough fragments are identified and then the method would look for another match.

The quality of the annotation determined here can be compared to an existing study [41]. In that study, peptides from the total hydrolysate of 20 % WPI hydrolyzed by Alcalase at the gelation point (after 5 hours of hydrolysis) were annotated using an automated method. No report of the quality of the annotation is present in that study. Nevertheless, the sequence coverage can be calculated from the list of annotated peptides provided. The calculated amino sequence coverage is 98 % and the peptide sequence coverage is 90 % with 130 peptides annotated at one DH. These values are comparable to those that were obtained with our method and indicate that a quite complete annotation can be obtained, even with an a-specific enzyme. In the study mentioned, comparable to the results presented above, cleavages are occurring after a large number of amino acids, principally Leu and Glu as well as aromatic acid residues. Unfortunately no information on the quantification was available in that study for further comparison.

Concluding remarks

In this study, the influence of different parameters could only be studied after developing the tools to characterize the hydrolysis and its mechanism. The selectivity

was developed as a global parameter of the mechanism of hydrolysis. This parameter enables the translation of a large set of peptide annotation and quantification data into a value of enzyme preference. Using this parameter, it was determined that the cleavage sites in a protein substrate are not hydrolyzed equally. In addition, changing the conditions of hydrolysis resulted in changes in kinetics and in the mechanism of hydrolysis. Finally, using the methods developed in this thesis, the control of the hydrolysis towards the formation of specific peptides can be considered. Also, by increasing the complexity, i.e. number of substrate molecules and a-specific enzymes, more complex systems can be tackled. With this, understanding dynamic *in-vitro* and even *in-vivo* digestion of proteins can be considered.

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Annex 1. List of peptides derived from β-lactoglobulin identified in all hydrolysates of (0.1-10 % (w/v)) WPI by BLP. (* indicates a-specific peptides) - (chapter 5).

fragment	sequence	theoretical mass (Da)	mass observed (Da)	Error (ppm)	charge state	b fragments identified	y fragments identified
[1-11]	LIVTQTMKGLD	1217.669	1217.686	14	2+	2,5	4,5,6,7,8,9
[1-28]	LIVTQTMKGLDIQKVAGTWYSLAMAASD	3010.550	3010.557	2	2+	4,10	
[1-33]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLD	3551.862	3551.841	-6	3+, 2+	2,7	1,7,8
[1-45]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	4894.550	4894.611	12	3+, 4+	2	1,4,7,8,14,23(2+)
[1-51]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPE	5559.925	5559.768	-28	4+,	1,2,3,4,12	1,2,7,21(2+),22(2+),26(2+)
[1-55]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPE GDLE	5974.099	5973.968	-22	3+,4+	1,2,21(2+),23(2+)	1,3,14(2+),18(2+),21(2+), 24(2+)
[1-62]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLD AQSAPLRVYVEELKPTPEGDLEILLQKWE	6884.628	6884.44	-27	4+	1,2	5,30(2+)
[12-45]	(D)-IQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEE	3694.892	3694.836	-15	3+,	1,7,11,14,18,22(2+)	5,8,21
[29-45]	(D)-ISLLDAQSAPLRVYVEE	1902.009	1902.028	10	3+,4+	2,5,7,8,9,11,12,13,14,15,16	2,3,5,6,8,10,17
[34-45]	(D)-AQSAPLRVYVEE	1360.698	1360.718	15	2+,1+	2,3,8,9,10,11	1,6,8,9,10,12
[36-45]	(Q)-SAPLRVYVEE*	1161.603	1161.606	3	2+,1+	2,3,7,8,9	6,7,8,10
[46-51]	(E)-LKPTPE	683.385	683.397	18	2+,1+	5	2,3,4,5,6
[46-54]	(E)-LKPTPEGDL*	968.518	968.526	8	1+		3,8
[46-55]	(E)-LKPTPEGDLE	1097.560	1097.57	9	2+,1+	2,4,6,8,9	1,6,7,8,9,10
[52-55]	(E)-GDLE	432.186	432.189	7	1+	3,4	1
[56-59]	(E)-ILLQ*	485.321	485.339	37	1+	2,3	1,2
[56-62]	(E)-ILLQKWE	928.538	928.537	-1	2+,1+	5,6	2,3,4,5,6,7
[56-65]	(E)-ILLQKWENGE	1228.645	1228.634	-9	2+	2	6,7,8
[56-74]A	(E)-ILLQKWENDECAQKKIIAE	2271.193	2271.194	0	3+,2+	2,5	2,5,6,9
[60-62]	(Q)-KWE*	461.227	461.231	9	1+	1,2,3	2
[63-74]A	(E)-NDECAQKKIIAE	1360.666	1360.684	13	2+,1+	2,3,4,5,6,8,9,10	1,2,3,4,5,7,8,9,10,12
[63-74]B	(E)-NGECAQKKIIAE	1302.660	1302.672	9	2+,1+	2,3,8,9,10	1,2,6,7,8,9,12
[63-89]A	(E)-NDECAQKKIIAEKTKIPAVFKIDALNE	3028.627	3028.635	3	3+,2+	1,2,3	11,15(2+),27(2+)
[63-89]B	(E)-NGECAQKKIIAEKTKIPAVFKIDALNE	2970.621	2970.615	-2	4+,3+,2+	1,11	4,7,11,15(2+),24(2+),27(2+)
[66-74]	(E)-CAQKKIIAE	1002.553	1002.560	7	2+,1+	1,2,3,5,6,7,8,	1,2,5,6,7
[66-108]	(E)-CAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCME	4986.704	4986.711	1	4+,3+		1,2,3,4,16(2+),17(2+)
[75-80]	(E)-KTKIPA*	656.422	656.435	20	1+	1,2,3,4,6	2,5
[75-85]	(E)-KTKIPAVFKID	1258.765	1258.772	6	2+	1,2,3,4,7,8,9	1,7,8
[75-89]	(E)-KTKIPAVFKIDALNE	1685.972	1685.974	1	3+,2+,1+	1,2,3,4,6,7,8,11	7,8,11
[75-108]	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCME	4002.161	4002.153	-2	4+,3+	2,7	
[75-131]B	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQC LVRTPEVDDE	6516.262	6516.452	29	4+	1,8,21(2+)	1,3,6
[75-134]B	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQC LVRTPEVDDEALE	6829.425	6829.348	-11	4+		2,5,6,9
[81-89]	(A)-VFKIDALNE*	1047.560	1047.582	21	2+,1+	5,6,7,8	5,6,7,8,9
[86-89]	(D)-ALNE	445.217	445.224	16	1+	2,3	1,2,4
[90-108]	(E)-NKVLVLDTDYKKYLLFCME	2334.200	2334.208	3	3+,2+	2,3,4,5,7,8,9,14(2+), 15(2+),16(2+),17(2+),18(2+)	1,2,3,4,15(2+),16(2+),17(2+)
[90-114]	(E)-NKVLVLDTDYKKYLLFCMENSAEPE	2961.450	2961.429	-7	3+,2+	1	1,2,25(2+)
[90-127]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPE	4418.167	4418.164	-1	3+,4+	2,3,4,5,6	1,2,3,4,5,7

Annex 1. Continued.

fragment	sequence	theoretical mass (Da)	mass observed (Da)	Error (ppm)	charge state	b fragments identified	y fragments identified
[86-89]	(D)-ALNE	445.217	445.226	20	1+	2,3	1,2,4
[90-108]	(E)-NKVLVLDTDYKKYLLFCME	2334.200	2334.198	-1	3+,2+	2,3,4,5,7,8,9,14(2+),15(2+), 16(2+),17(2+),18(2+)	1,2,3,4,15(2+),16(2+),17(2+)
[90-127]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPE	4418.167	4418.214	11	3+,4+	2,3,4,5,6	1,2,3,4,5,7
[90-127]B	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPE	4390.136	4390.179	10	3+,4+	2,3,4,5,6	4,5,25(3+)
[90-131]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDE	4876.332	4876.386	11	3+,4+	5,6,36(3+)	1,3,6
[90-134]A	(E)-NKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALE	5189.495	5189.520	5	3+,4+	1	1,2,6,9
[97-108]	(D)-TDYKKYLLFCME	1552.730	1552.746	10	2+	4,8,9	2,3,4,5,6,8
[109-127]A	(E)-NSAEPEQSLVCQCLVRTPE	2101.977	2101.988	5	2+,3+	2,3,4,6,7,8,9,12,13,14,15, 16,17	2,3,4,5,6,7,8,9,10,11,15
[109-127]B	(E)-NSAEPEQSLACQCLVRTPE	2073.946	2073.952	3	2+,3+	4,13,14,15,16	2,4,5,7,11,12,13,14,15
[109-131]A	(E)-NSAEPEQSLVCQCLVRTPEVDDE	2560.142	2560.154	5	2+,3+	4,11,13	6,7,8,10,11,13
[109-131]B	(E)-NSAEPEQSLACQCLVRTPEVDDE	2532.111	2532.136	10	2+,3+	7,11,14	1,12,14
[109-134]A	(E)-NSAEPEQSLVCQCLVRTPEVDDEALE	2873.306	2873.316	3	3+,2+	1,2,3,4,6,7,10,12,15	1,2,6,9,10,11
[115-127]A	(E)-QSLVCQCLVRTPE	1474.727	1474.742	10	2+	1,3,4,9,10,11	2,3,4,5,7,8,9,10,11
[115-127]B	(E)-QSLACQCLVRTPE	1446.695	1446.712	12	2+	2,3,4,8,10,11	2,4,5,7,8,9,10,11,12,13
[115-134]B	(E)-QSLACQCLVRTPEVDDEALE	2218.025	2218.028	1	2+	1,5,11	2,10
[128-134]	(E)-VDDEALE	789.339	789.347	10	1+	3,5,6,7	
[135-137]	(E)-KFD	408.201	408.211	24	1+	1,2	1,2,3
[135-141]	(E)-KFDKALK*	848.512	848.519	8	2+,1+	1	1,6,7
[135-157]	(E)-KFDKALKALPMHIRLSFNPTQLE	2696.484	2696.478	-2	3+,4+,2+	1,2,4,5,6,9,11,17(2+), 18(2+),20(2+)	1,2,3,5,6,9,11,20(2+), 21(2+),22(2+)
[135-158]	(E)-KFDKALKALPMHIRLSFNPTQLEE	2825.526	2825.520	-2	3+,4+,2+	1,2,3,4,5,6,9	1,2,3,4,5,6,7,9,11,12,13,15
[135-162]	(E)-KFDKALKALPMHIRLSFNPTQLEEQCHI	3306.737	3306.741	1	3+,4+,2+	1,3,4	2,3,4,5,6,7,8,21(2+),25(2+)
[138-157]	(D)-KALKALPMHIRLSFNPTQLE	2306.293	2306.260	-14	2+,1+	3,5,17(2+),18(2+)	6
[138-158]	(D)-KALKALPMHIRLSFNPTQLEE	2435.336	2435.350	6	3+,2+	3,4,10,15(2+),18(2+),19(2+)	1,6,8
[142-157]	(K)-ALPMHIRLSFNPTQLE*	1865.982	1865.990	4	2+,3+	3,5,11,15(2+)	2,3,5,6,14(2+)
[142-158]	(K)-ALPMHIRLSFNPTQLEE*	1995.025	1995.010	-8	2+,3+	3,5,11(2+),14(2+),15(2+),16 (2+)	6,7,15(2+)
[146-157]	(M)-HIRLSFNPTQLE*	1453.768	1453.768	0	2+,	2,3,4,5,6,7,9	1,2,3,6,8,10
[146-158]	(M)-HIRLSFNPTQLEE*	1582.810	1582.814	3	2+	2,3,6,7,9	1,6,7,11
[158-162]	(E)-EQCHI	628.264	628.270	10	1+	4,5	2,3,4
[159-162]	(E)-QCHI	499.221	499.229	16	1+	3,4	2

Annex 2. Peptides derived from β-lactoglobulin identified in all hydrolysates of 1 % (w/v) WPI by BLP at pH 7-9, in addition to peptides in Annex 1. (* indicates a-specific peptides) - (chapter 6).

fragment	sequence	theoretical mass (Da)	mass observed (Da)	Error (ppm)	charge state	b fragments identified	y fragments identified
[1-44]	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVE	4765.507	4765.47	-8	3+,4+	2,3,5,7,24(2+),26(2+),28(2+)	16(2+),24(2+),25(2+)
[45-55]	(E)-ELKPTPEGDLE	1226.603	1226.612	7	1+,2+	9,10	6,8,9,10,11
[56-85]B	(E)-ILLQKWENGECAQKKIIAEKTKIPAVFKID	3453.942	3453.768	-50	2+,3+	2	6,13,30(2+)
[75-127]A	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAE PEQSLVCQCLVRTPE	6086.128	6086.020	-18	4+,5+	1,21(2+),34(2+)	2,4,15(2+)
[75-134]A	(E)-KTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSA EPEQSLVCQCLVRTPEVDDEALE	6857.457	6857.460	1	4+,5+	1,4	2,3,6,9
[81-85]*	(A)-VFKID	620.353	620.356	5	1+	4	5
[86-96]	(D)-ALNENKVLVLD	1226.687	1226.592	-77	2+		11
[99-129]B	(D)-YKKYLLFCMENSAEPEQSLACQCLVRTPEVD	3606.687	3606.908	61	3+,4+	4,7	
[135-145]*	(E)-KFDKALKALPM	1260.726	1260.726	0	2+,1+	1,3,4,5,6,7,8,9	2,9,11
[135-150]*	(E)-KFDKALKALPMHIRLS	1867.087	1867.068	-10	3+,2+	1,3,6,9	5,6,7,8
[151-162]*	(S)-FNPTQLEEQCHI	1457.661	1457.698	25	2+	9(2+),10(2+)	

Annex 3. Concentration (μM) of peptides annotated in 0.1-10 % (w/v) WPI hydrolyzed by BLP (chapter 5). All hydrolysates were diluted to 0.1 % (w/v) prior to analysis.

	0.1% WPI					0.5 % WPI					1 % WPI					5 % WPI					10 % WPI				
	DH (%)					DH (%)					DH (%)					DH (%)					DH (%)				
	1.5	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**
[90-127]A		0.224	1.73	5.69		1.01	3.84	4.24	3.19		1.59	4.92	1.83				4.62	14.7	8.02	1.96		2.09	1.54	4.25	4.42
[90-127]B					0.206							4.28	1.13									5.24			
[90-131]A													0.082					0.590				1.24	1.05		
[90-134]A						1.24	0.820				4.09	2.03				2.72	3.36				6.82	3.94			
[90-134]B																		2.42			2.55				
[97-108]													0.388	3.14	5.60			0.671	2.20	2.47				2.58	
[109-127]A	0.95	1.72	4.11	5.37	5.01	0.490	2.26	4.48	3.75	2.10		2.26	6.19	4.22	0.989		0.805	3.05	3.40	0.11		0.542	1.61	2.60	5.08
[109-127]B	2.86	2.49	6.38	14.7	16.8	1.32	6.53	15.7	15.3	5.65	4.31	0.412					0.338	0.640				1.79	0.581	0.644	
[109-129]B																			5.46					11.7	0.782
[109-131]A			0.185	0.440			0.263	0.768				0.667	1.58					2.22				0.649	0.962		0.693
[109-131]B												0.268	2.84				0.856	1.83				0.875	1.31		
[109-134]A	5.51	4.51	1.99	0.521		2.44	3.38	0.663				2.98				0.914	2.12	1.11			0.832	1.51	1.23		
[109-134]B							0.468									0.921	1.15								
[113-157]A																						0.114			
[115-127]A			1.20	2.94	4.56	0.301	0.560	3.30	7.15	12.4		1.33	4.11	12.9	16.0		0.267	1.68	9.12	11.0			1.02	10.7	7.52
[115-127]B	1.03	0.626	2.08	2.66	2.93		1.48	2.83	7.85	10.6		1.33	5.01	9.05	6.74		1.10	4.25	8.38	4.61		1.21	2.94	9.73	11.7
[115-131]A				0.535	0.719			0.803	0.486									0.556				0.490	0.820	1.08	0.995
[115-131]B									0.337	1.51													0.402	0.301	2.01
[115-134]A		1.07	1.82	2.74	4.11	0.719	2.87	5.57	3.84																
[115-134]B				0.510	0.740		0.402	0.672				1.74	1.45				1.62	0.823				0.743	0.742		
[128-134]	7.16	9.67	13.0	24.0	24.7	4.95	13.1	23.6	26.8	23.2	6.33	15.0	27.3	18.1	5.57	3.26	11.6	15.5	2.79		3.03	8.27	13.3		
[135-137]														2.57	7.62				3.18	2.56				4.23	1.28
[135-141]															1.65										
[135-157]	3.16	3.76	3.85	4.76	5.11	1.33	2.84	3.08	5.56	8.42	0.792	0.565	0.783	4.77	2.49		0.474	0.483	4.28	0.154		0.418	0.547	0.710	0.677
[135-158]	8.18	8.24	11.5	17.4	19.9	6.23	14.5	25.3	33.8	24.5	6.99	14.1	21.2	7.07	1.88	6.99	13.6	16.5	10.2	0.578	6.90	14.0	17.9	3.76	13.0
[135-162]	12.0	6.03	6.05	4.36	3.24	7.74	6.91	3.85	1.49	1.79	13.9	8.51	1.29	0.702		11.1	4.43	1.72	1.51		7.90	4.25	1.82		
[138-145]																			1.18	10.3					
[138-157]														0.451	0.720				1.38	2.38				1.57	
[138-158]									0.295					1.23	0.655				2.25	0.907				2.17	1.44
[138-162]																			1.16						
[142-157]															1.04										
[142-158]									0.291	0.315				1.29	2.09										
[146-150]																				5.92					
[146-157]															3.82					3.58					
[146-158]														2.53	4.96				1.35	2.49					
[151-157]																				4.99				1.36	
[151-158]																				1.10				1.50	
[158-162]	1.14	0.579	1.12	0.843	0.961	1.68	0.659	0.646	0.895	0.542	1.99	3.79	4.01	3.52	3.08	1.81	3.04	2.34	2.70	2.34	1.42	2.27	2.35	2.66	2.87
[159-162]	11.2	7.37	13.3	16.2	20.5	8.87	15.3	24.7	30.2	25.9	11.9	20.3	28.8	29.5	31.2	10.8	21.3	27.9	32.2	25.3	11.7	24.5	30.1	32.1	42.3

Annex 3. continued

	0.1% WPI					0.5 % WPI					1 % WPI					5 % WPI					10 % WPI				
	DH (%)					DH (%)					DH (%)					DH (%)					DH (%)				
	1.5	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**
[90-127]A		0.224	1.73	5.69		1.01	3.84	4.24	3.19		1.59	4.92	1.83				4.62	14.7	8.02	1.96		2.09	1.54	4.25	4.42
[90-127]B					0.206							4.28	1.13									5.24			
[90-131]A													0.082					0.590				1.24	1.05		
[90-134]A						1.24	0.820				4.09	2.03				2.72	3.36				6.82	3.94			
[90-134]B																		2.42			2.55				
[97-108]													0.388	3.14	5.60			0.671	2.20	2.47				2.58	
[109-127]A	0.95	1.72	4.11	5.37	5.01	0.490	2.26	4.48	3.75	2.10		2.26	6.19	4.22	0.989		0.805	3.05	3.40	0.11		0.542	1.61	2.60	5.08
[109-127]B	2.86	2.49	6.38	14.7	16.8	1.32	6.53	15.7	15.3	5.65	4.31	0.412					0.338	0.640				1.79	0.581	0.644	
[109-129]B																			5.46					11.7	0.782
[109-131]A			0.185	0.440			0.263	0.768				0.667	1.58					2.22				0.649	0.962		0.693
[109-131]B												0.268	2.84				0.856	1.83				0.875	1.31		
[109-134]A	5.51	4.51	1.99	0.521		2.44	3.38	0.663				2.98				0.914	2.12	1.11			0.832	1.51	1.23		
[109-134]B							0.468									0.921	1.15								
[113-157]A																						0.114			
[115-127]A			1.20	2.94	4.56	0.301	0.560	3.30	7.15	12.4		1.33	4.11	12.9	16.0		0.267	1.68	9.12	11.0			1.02	10.7	7.52
[115-127]B	1.03	0.626	2.08	2.66	2.93		1.48	2.83	7.85	10.6		1.33	5.01	9.05	6.74		1.10	4.25	8.38	4.61		1.21	2.94	9.73	11.7
[115-131]A				0.535	0.719			0.803	0.486									0.556				0.490	0.820	1.08	0.995
[115-131]B									0.337	1.51													0.402	0.301	2.01
[115-134]A		1.07	1.82	2.74	4.11	0.719	2.87	5.57	3.84																
[115-134]B				0.510	0.740		0.402	0.672				1.74	1.45				1.62	0.823				0.743	0.742		
[128-134]	7.16	9.67	13.0	24.0	24.7	4.95	13.1	23.6	26.8	23.2	6.33	15.0	27.3	18.1	5.57	3.26	11.6	15.5	2.79		3.03	8.27	13.3		
[135-137]														2.57	7.62				3.18	2.56				4.23	1.28
[135-141]															1.65										
[135-157]	3.16	3.76	3.85	4.76	5.11	1.33	2.84	3.08	5.56	8.42	0.792	0.565	0.783	4.77	2.49		0.474	0.483	4.28	0.154		0.418	0.547	0.710	0.677
[135-158]	8.18	8.24	11.5	17.4	19.9	6.23	14.5	25.3	33.8	24.5	6.99	14.1	21.2	7.07	1.88	6.99	13.6	16.5	10.2	0.578	6.90	14.0	17.9	3.76	13.0
[135-162]	12.0	6.03	6.05	4.36	3.24	7.74	6.91	3.85	1.49	1.79	13.9	8.51	1.29	0.702		11.1	4.43	1.72	1.51		7.90	4.25	1.82		
[138-145]																			1.18	10.3					
[138-157]														0.451	0.720				1.38	2.38				1.57	
[138-158]										0.295				1.23	0.655				2.25	0.907				2.17	1.44
[138-162]																			1.16						
[142-157]															1.04										
[142-158]									0.291	0.315				1.29	2.09										
[146-150]																				5.92					
[146-157]															3.82					3.58					
[146-158]														2.53	4.96				1.35	2.49					
[151-157]																				4.99				1.36	
[151-158]																				1.10				1.50	
[158-162]	1.14	0.579	1.12	0.843	0.961	1.68	0.659	0.646	0.895	0.542	1.99	3.79	4.01	3.52	3.08	1.81	3.04	2.34	2.70	2.34	1.42	2.27	2.35	2.66	2.87
[159-162]	11.2	7.37	13.3	16.2	20.5	8.87	15.3	24.7	30.2	25.9	11.9	20.3	28.8	29.5	31.2	10.8	21.3	27.9	32.2	25.3	11.7	24.5	30.1	32.1	42.3

Annex 4. Concentration (µM) of peptides annotated for 1 % (w/v) WPI hydrolyzed by BLP at different pH of hydrolysis (chapter 6). All hydrolysates were diluted to 0.1 % (w/v) prior to analysis.

	pH7				pH 8					pH9				
	DH (%)													
	1.5	**3**	**4.5**	**6**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**
[1-11]				0.350					2.24					0.896
[1-28]			0.478	0.520					0.248				0.299	0.939
[1-33]				0.927				0.700	1.05				0.701	0.780
[1-44]			0.293	0.595										
[1-45]	4.19	8.52	10.00	7.10	10.2	14.5	12.8	7.32	1.90	7.95	14.8	18.2	13.4	7.55
[1-51]					0.245									
[1-55]	0.592	0.413			3.24	0.775				2.15	0.427			
[1-62]	0.314				0.299									
[1-162]	29.4	19.2	18.7	2.64	17.1	3.60				8.69	1.13			
[12-45]												0.374	0.543	1.26
[29-45]			0.289	0.745				1.65	2.19			0.329	0.962	1.94
[34-45]			1.24	3.90			1.13	7.76	13.7			0.774	2.91	6.40
[36-45]													1.45	3.54
[45-55]				0.668										
[46-51]	0.939	2.96	7.51	22.0	1.17	3.44	6.96	23.2	30.6	2.05	5.52	12.5	44.9	74.6
[46-54]				0.622										
[46-55]	8.68	17.8	24.7	21.1	21.49	31.3	35.0	14.6	2.44	16.4	29.9	30.8	19.7	8.19
[52-55]		2.38	5.07	13.6		4.00	6.58	16.6	24.2			4.94	15.1	24.8
[56-59]						2.22	3.22	8.46	14.8		2.15	6.50	20.9	29.4
[56-62]	5.38	11.2	14.3	18.4	15.7	27.0	33.0	26.2	15.0	9.71	17.0	14.8	4.46	
[56-65]B										0.395	0.443			
[56-74]A	0.437	0.307			1.49	0.647				1.52				
[56-85]B													0.974	
[60-62]						0.457	1.18	7.08	11.7			3.818	14.3	17.3
[63-74]A	3.90	9.93	11.3	3.9	7.90	13.5	12.8	4.34	2.92	4.67	11.6	11.5	3.00	0.951
[63-74]B	0.553	1.98	4.18	4.18	2.08	4.87	8.15	5.13	1.45	1.00	4.83	9.25	4.60	0.61
[63-89]A	0.758	1.15	0.375		2.69	3.52	0.673			1.50	1.88	0.218		
[63-89]B	0.224						0.496			0.545	1.96	0.840		
[63-108]A					2.26					0.568				
[66-74]	0.865	2.80	10.8	23.8		3.44	13.2	24.9	28.4	1.22	2.75	9.49	23.5	33.0
[66-89]		0.195					0.316							
[75-80]			5.02	30.7			1.35	9.24	21.6					
[75-85]			0.519					0.995	5.82				0.515	1.42
[75-89]	3.71	11.6	20.9	6.59	5.86	15.0	30.2	23.7	12.5	4.76	19.8	32.8	36.5	34.3
[75-108]	2.57	3.81	2.14	0.97	4.81	9.00	1.70			6.01	5.03	2.96		
[75-127]A	0.581	1.49	0.30							0.834	0.726			
[75-134]A	1.07	1.09								3.08	1.54			
[75-134]B					0.946	0.303				1.11				
[81-85]				1.72										
[81-89]			4.34	23.1				5.34	3.82					
[86-89]		2.65	0.926	2.42				3.86	6.73				2.75	2.80
[86-96]			1.09											
[90-108]	3.43	8.68	17.8	26.1	6.21	7.90	19.1	15.5	15.0	4.10	13.3	23.2	25.8	28.0
[90-114]		0.212	0.591	0.663							0.335	0.651	0.456	0.357
[90-127]A	1.46	3.18	3.44	1.56	1.59	4.92	1.83			0.949	1.60	1.94	2.48	1.91
[90-127]B						4.28	1.13					1.41		
[90-131]A							0.082					0.880		
[90-134]A	1.59	0.616			4.09	2.03				4.44	1.81			
[90-134]B	0.672									1.26	2.17			
[97-108]							0.388	3.14	5.60					0.264
[99-129]B			3.01	2.39										
[109-127]A	0.690	3.45	6.94	4.12		2.26	6.19	4.22	0.989	0	2.84	4.56	3.03	1.46
[109-127]B	2.49	5.60	11.3	9.75	4.31	0.412				4.05	9.79	14.9	5.62	1.92
[109-131]A	0.253	1.22	1.55			0.667	1.58				0.712	1.29		
[109-131]B	0.433	0.737	1.76			0.268	2.84					1.89	0.267	
[109-134]A	1.45	1.28	0.390			2.98				2.71	3.79	1.12		
[109-134]B										0.993				
[115-127]A	0.745	1.14	5.09	6.92		1.33	4.11	12.9	16.0	0	0.800	3.73	10.4	15.2
[115-127]B		1.09	2.88	5.24		1.33	5.01	9.05	6.74		0.765	4.88	10.60	11.93

Annex 4. Continued.

	pH7				pH 8					pH9				
	DH (%)													
	1.5	**3**	**4.5**	**6**	**1.5**	**3**	**4.5**	**6**	**7**	**1.5**	**3**	**4.5**	**6**	**7**
[115-131]A			0.919	0.206								0.933	1.15	
[115-131]B												0.405	2.45	0.921
[115-134]A	0.615	0.920	0.577							0.627	1.67	1.50	0.047	0.058
[115-134]B		0.622				1.74	1.45				1.41	1.60		
[128-134]	2.39	6.61	11.5	11.2	6.33	15.0	27.3	18.1	5.57	2.31	8.45	15.2	13.6	6.92
[135-137]			0.757	2.81				2.57	7.62				1.53	3.51
[135-141]			0.709	5.38					1.65					
[135-145]		0.671	2.52	8.94										
[135-150]		0.297	0.742	1.24										
[135-157]	1.57	3.05	4.94	3.37	0.792	0.565	0.783	4.77	2.49	1.39	1.78	2.68	4.12	5.12
[135-158]	3.93	9.14	7.80	1.77	6.99	14.1	21.2	7.07	1.88	4.84	13.2	21.0	22.5	19.4
[135-162]	6.94	4.66	1.50	0.702	13.9	8.51	1.29	0.702		17.2	12.7	5.58	2.68	1.66
[138-145]				2.02										
[138-157]			0.269	0.263				0.451	0.720				0.188	0.485
[138-158]			0.220	0.292				1.23	0.655			0.380	1.71	3.70
[142-157]			0.803	3.74					1.04					
[142-158]			0.757	1.55				1.29	2.09				0.233	0.292
[146-150]			0.193	4.17										
[146-157]		0.350	1.01	4.21					3.82					
[146-158]		0.540	1.51	3.17				2.53	4.96				1.13	0.244
[151-157]			0.743	5.41										0.279
[151-158]			1.29	3.93									1.07	2.41
[151-162]				0.294										
[158-162]	2.43	5.40	9.22	12.4	1.99	3.79	4.01	3.52	3.08	0.674	2.11	3.00	3.33	4.00
[159-162]	5.23	12.8	19.3	18.3	11.9	20.3	28.8	29.5	31.2	6.47	16.2	24.9	28.8	30.0

Summary

Enzymatic protein hydrolysates are widely used in the food industry for their techno-functional properties and their nutritional value. To understand the differences in peptide composition in protein hydrolysates that result from variations in hydrolysis conditions (e.g. enzymes, or substrate concentration) the mechanism of hydrolysis needs to be understood in detail. The five commonly used descriptors of hydrolysis and hydrolysates are discussed in **chapter 1**. These descriptors are the degree of hydrolysis (DH), the proportion of remaining intact protein, the molecular weight distribution of the peptides present, the identification of peptides and finally the quantification of peptides. Most published studies only use a few of these descriptors to describe the influence of the hydrolysis conditions and do not present a complete overview. In addition, the identification and quantification of peptides is not commonly applied to all peptides present in the hydrolysates. Hence, due to the lack of coherent information, the mechanism of hydrolysis, including the effects of substrate concentration and pH, is still poorly understood.

In **chapter 2**, the effect of increasing protein concentration (1-30 % (w/v)) on the kinetics of hydrolysis of WPI was compared for the a-specific enzymes Alcalase and Neutrase. For both enzymes, the increase in protein concentration at constant enzyme to substrate ratio resulted in a lower overall hydrolysis rate, as well as a lower DH reached after two hours. This confirmed the notion obtained from literature data that the observed effect is generic and does not depend on the enzyme used. To understand which factors influence the hydrolysis, the hydrolyses were performed at increased conductivity, in the presence of 0.5 M NaCl, conductivity was comparable to the one of 30 % (w/v) WPI (46 $mS \cdot cm^{-1}$). In addition, for 1 % with NaCl and for 30 % (w/v) WPI a similar final DH was reached. This indicated that the ionic strength of the solution is an important factor influencing the rate of hydrolysis.

In addition to the changes in ionic strength, also the amount of water in the system is significantly decreased at higher substrate concentrations (e.g. 30 % (w/v) WPI). While it seems obvious, the role of water is not often taken into consideration. To evaluate the water availability, the water activity was measured at the beginning of hydrolysis and after two hours of hydrolysis (**chapter 3**). The fraction of free water, calculated based on the hydration of individual amino acid residues is 0.99 for 0.1 % (w/v) WPI and 0.83 for 30 % (w/v) WPI. In addition, the fraction of free water was also determined by measuring the relaxation rate of water by NMR. From these experiments the fraction of free water was determined to be 0.9 at 30 % (w/v) WPI. These results confirmed the decrease in free water calculated. To evaluate the excess of water during the enzymatic hydrolysis the free to bound water ratio was determined as a function of the degree of hydrolysis. By increasing the protein concentration a decrease in the free to bound water ratio was observed, synonym to a decrease in water availability. The

decrease in the local rate of hydrolysis with increasing substrate concentration was correlated to this decrease in water availability.

While the kinetics of hydrolysis was affected by water availability, there may also be changes in mechanism. Since many peptides are formed, it is challenging to describe the hydrolysis process, and subsequently to evaluate the effects of changes in hydrolysis conditions. To characterize the mechanism of hydrolysis, the term *enzyme selectivity* was introduced as a new quantitative descriptor (**chapter 4**). The enzyme selectivity was defined as the relative rate of selective hydrolysis of a cleavage site compared to all cleavage sites in the sequence of the protein, given the reported specificity of the enzyme. The selectivity was determined using the enzyme *Bacillus licheniformis* protease (BLP), which is specific for glutamic acid and aspartic acid residues. To calculate the selectivity, the peptides obtained at different degrees of hydrolysis from hydrolysis of β-lactoglobulin were annotated and quantified. In addition to the development of the method for the calculation of the selectivity, new tools were defined to describe the quality of the peptide annotation and of the peptide quantification. The peptide sequence coverage is the quality parameter on the identification. It is an indication of the number of amino acids annotated over the total number of amino acids in annotated and missing peptides. The molar sequence coverage is the quality parameter for quantification. It indicates if all amino acids were quantified to the expected concentration. The determination of the selectivity showed that the selectivity towards the different cleavage sites of β-lactoglobulin by BLP vary from 0.003 % to 17 %, indicating large differences of the enzyme selectivity towards the cleavage sites after the same type of amino acids. With the selectivity, the enzyme action can be quantitatively described and the influence of the conditions of hydrolysis can be studied.

The methodology and tools developed in chapter 4 were used to study the influence of the substrate concentration on the mechanism of hydrolysis (**chapter 5**). As described in the earlier chapters, the overall hydrolysis rate is decreased with the increase in substrate concentration (0.1-10 % (w/v) WPI). The accessibility of the substrate to the enzyme is comparable for all protein concentrations. The selectivity was determined as the relative rate of selective hydrolysis for each cleavage site compared to all cleavage sites in the protein. A clear influence of the substrate concentration on four cleavage sites for which the enzyme has a very high selectivity (± 15 % as determined in the previous chapter) was noted. At 10 % (w/v) WPI the selectivity of two of these cleavage sites is increased by a factor 2 compared to the other substrate concentrations. For the two others, the enzyme selectivity is decreased by a factor 2. In addition, the enzyme had a higher selectivity towards Asp residues at high protein concentration (1-10 % (w/v) WPI) than at low protein concentrations (0.1-0.5 % (w/v) WPI). This indicates that both the selectivity and the overall hydrolysis rate are changed with increasing substrate concentration.

In **chapter 6**, the influence of the pH of hydrolysis on the selectivity was determined. For the hydrolysis of 1 % (w/v) WPI by BLP, the overall hydrolysis rate is increased with

the increase in pH from 7.0 to 9.0. From literature it is known that the stability of β-lactoglobulin decreases with the same increase of pH. Consequently, the accessibility of intact β-lactoglobulin towards hydrolysis increased with the decrease in protein stability. As further indication of changes in mechanism, peptides were identified and quantified for the different conditions as a function of the DH. It was found that there is more accumulation of the intermediate peptides (i.e. peptides formed during hydrolysis that can be further hydrolyzed) at pH 9.0 compared to pH 7.0. The selectivity shows five different types of behavior as a function of the pH for all cleavage sites in β-lactoglobulin. Significant changes were observed in the selectivity with either minimum values at pH 8.0 or large increase and decrease as a function of the pH. In conclusion, changing the pH of hydrolysis influences the overall hydrolysis rate and the mechanism of hydrolysis.

During peptide identification for the determination of the enzyme selectivity, a number of unexpected peptides, based on the enzyme specificity were annotated. It was found that the formation of these a-specific peptides results from the cleavage of previously formed specific peptides and not from direct hydrolysis of the intact protein. This was confirmed by incubating these peptides, produced synthetically, alone in the presence of the enzyme (**chapter 7**). In addition, by incubating the synthetized peptides without the enzyme, the same bonds were cleaved. This showed that not all peptides formed during protein hydrolysis are stable. The instability is further enhanced in the presence of the enzyme.

During the analysis of the hydrolysates a large variety of peptides is formed. By using the descriptor selectivity to characterize the mode of action of the enzyme, the large amount of data created is conveniently reduced. In addition to this experimental approach, a simulation model was used to describe the hydrolysis of proteins (**chapter 8**). This stochastic model is based on a random selection of substrate molecules and cleavage locations. The influence of the number of cleavage site and the distribution of the cleavage sites in the protein on the selectivity was demonstrated. In addition, a good correlation was obtained between simulated and experimental enzyme selectivity towards most of the cleavage sites. The fact that the selectivity for some cleavage sites cannot be simulated indicate that it is difficult to account for all the parameters that influence the selectivity.

In **chapter 9**, the challenges encountered for a complete description of the mechanism of hydrolysis are presented. This includes the difficulties in annotating and quantifying all peptides present in the hydrolysates. In addition, the parameters developed to identify the quality of the annotation and quantification are discussed. Finally, the determination and applicability of the newly defined parameter selectivity are discussed. With this parameter it was clearly established that there are large differences in the rate of hydrolysis of the different cleavage sites by the enzyme. The parameters developed for a simple system in this project are considered applicable to determine the enzyme selectivity in more complex systems, with more protein as substrates or for a-specific enzymes.

Samenvatting

Enzymatische eiwithydrolysaten worden wereldwijd toegepast in de levensmiddelenindustrie vanwege hun techno-functionele eigenschappen en voedingswaarde. Om te begrijpen hoe verschillen in de peptidesamenstelling van eiwithydrolysaten ontstaan door variaties in hydrolyse condities (bijv. enzym of substraatconcentratie) moet het mechanisme in detail worden begrepen. De hydrolyse en hydrolysaten worden vaak beschreven met vijf parameters, die worden besproken in **hoofdstuk 1**. Deze parameters zijn de hydrolysegraad (DH), de verhouding intact gebleven eiwit, de molecuulgewichtsverdeling van de aanwezige peptiden, de identificatie van peptiden en ten slotte de kwantificatie van peptiden. De meeste gepubliceerde werken gebruiken maar enkele van deze parameters om de invloed van hydrolysecondities te beschrijven en geven geen compleet overzicht. Daarnaast worden in het algemeen niet alle aanwezige peptiden in de hydrolysaten geïdentificeerd en gekwantificeerd. Door dit tekort aan coherente informatie wordt het mechanisme achter de hydrolyse, inclusief de effecten van substraatconcentratie en pH, nog steeds slecht begrepen.

In **hoofdstuk 2** wordt het effect van toenemende eiwitconcentratie (1-30 % (w/v)) op de kinetiek van hydrolyse van wei-eiwitisolaat (WPI) door de aspecifieke enzymen Alcalase en Neutrase vergeleken. Voor beide enzymen resulteerde de toename in eiwitconcentratie bij een constante enzym tot substraat verhouding zowel in een lagere hydrolysesnelheid als in een lagere DH na twee uur. Dit bevestigde de door literatuur gesteunde opvatting dat het waargenomen effect generiek is en niet afhankelijk is van het gebruikte enzym. Om te begrijpen welke factoren hydrolyse beïnvloeden, zijn de hydrolyses (bij 1-30 % w/v WPI) uitgevoerd in aanwezigheid van 0.5 M NaCl. De geleidbaarheid was voor alle oplossingen vergelijkbaar met die van 30 % (w/v) WPI (46 $mS.cm^{-1}$). Daarnaast werd een vergelijkbare DH bereikt voor beide series oplossingen. Hieruit bleek dat de ionsterkte van de oplossing een belangrijke factor is die de hydrolysesnelheid beïnvloedt.

Naast de veranderingen in ionsterkte is er ook een significante afname in hoeveelheid water in het systeem bij hogere substraatconcentraties (bijv. 30 % (w/v) WPI). Ondanks dat het zo voor de hand liggend lijkt, wordt er vaak geen rekening gehouden met de rol van water. Om de beschikbaarheid van water te bepalen werd de water activiteit gemeten aan het begin en het eind (na twee uur) van de hydrolyse (**hoofdstuk 3**). De fractie vrij water, berekend op basis van de hydratatie van individuele aminozuurresiduen, is 0.99 bij 0.1 % (w/v) WPI en 0.83 bij 30 % (w/v) WPI. Daarnaast werd ook de fractie vrij water bepaald door het meten van de relaxatiesnelheid van water met kernspinresonantie (NMR). In deze experimenten werd de fractie vrij water vastgesteld op 0.9 bij 30 % (w/v) WPI. Deze resultaten bevestigden de berekende afname van vrij water. Om het overtollige water tijdens de enzymatische hydrolyse te

bepalen werd de verhouding vrij : gebonden water vastgesteld als een functie van de hydrolysegraad. Door de eiwitconcentratie te verhogen werd een afname in de verhouding vrij : gebonden water geobserveerd, hetgeen gelijk staat aan een afname van waterbeschikbaarheid. De afname in de lokale hydrolysesnelheid bij toenemende substraatconcentratie is gecorreleerd aan deze afname aan waterbeschikbaarheid.

Hoewel de hydrolysekinetiek werd beïnvloed door de waterbeschikbaarheid, zijn er mogelijk ook veranderingen in het hydrolyse mechanisme. Omdat er veel peptiden worden gevormd, is het een uitdaging om het hydrolyse proces te omschrijven, en om de effecten op hydrolysecondities te voorspellen. Om het hydrolyseproces te karakteriseren, werd de term *enzymselectiviteit* geïntroduceerd als nieuwe kwantitatieve parameter (**hoofdstuk 4**). De enzymselectiviteit werd gedefiniëerd als als de relatieve snelheid van selectieve hydrolyse van een knippositie vergeleken met alle knipposities in de eiwitsequentie, voor algemeen beschreven enzymspecificiteit. De selectiviteit werd bepaald aan de hand van het enzym *Bacillus licheniformis* protease (BLP) dat een specificiteit heeft voor glutaminezuur- en asparaginezuurresiduen. Om de selectiviteit te kunnen berekenen, werden de peptiden - gevormd bij verschillende hydrolysegraden van β-lactoglobuline- geannoteerd en gekwantificeerd. Naast de ontwikkeling van een methode waarmee de selectiviteit berekend kan worden, werden nieuwe termen gedefiniëerd om de kwaliteit van de annotatie en de kwantificatie van de peptides te omschrijven. De dekkingsgraad van de peptidesequentie is de kwaliteitsparameter voor identificatie. Het is een indicatie van het aantal geannoteerde aminozuren ten opzichte van het totaal aantal aminozuren in geannoteerde en ontbrekende peptiden. De dekkingsgraad van de molaire sequentie is de kwaliteitsparameter voor kwantificatie. Deze term bescrhijft de experimenteel bepaalde concentratie van alle aminozuren ten opzichte van de verwachte concentratie. De bepaling van de selectiviteit liet zien dat de selectiviteit jegens de verschillende kniplocaties van β-lactoglobuline voor BLP varieerde van 0.003 % tot 17 %. Dit is een indicatie is voor grote verschillen van enzymselectiviteit jegens kniplocaties na hetzelfde type aminozuren. Met de selectiviteit kan de enzymwerking kwantitatief worden beschreven en de invloed van de hydrolysecondities worden bestudeerd.

De methodologie en technieken ontwikkeld in hoofdstuk 4 waren gebruikt in een studie naar de invloed van de substraatconcentratie op het hydrolyse mechanisme (**hoofdstuk 5**). Zoals in eerdere hoofdstukken is beschreven nam de hydrolyse snelheid af bij een toename in de substraatconcentratie (0.1-10 % (w/v) WPI). De beschikbaarheid van het substraat voor het enzym is vergelijkbaar bij alle eiwitconcentraties. De selectiviteit werd gedefinieerd als de relatieve snelheid van de selectieve hydrolyse voor elke kniplocatie ten opzichte van alle kniplocaties in het eiwit. Er werd een duidelijke invloed geconstateerd van de substraatconcentratie op vier kniplocaties waar het enzym een hoge selectiviteit voor had (± 15 % zoals werd bepaald in het vorige hoofdstuk). Bij 10 % (w/v) WPI neemt de selectiviteit van twee van deze kniplocaties met factor 2 toe, bij verhoging van de substraatconcentratie. Voor de twee andere kniplocaties nam de enzymselectiviteit af met een factor 2.

Tevens had het enzym een hogere selectiviteit voor asparaginezuurresiduen (Asp residuen) bij hoge eiwitconcentraties (1-10 % (w/v) WPI) dan bij lage eiwitconcentraties (0.1-0.5 % (w/v) WPI). Dit geeft aan dat zowel de selectiviteit als de algehele hydrolysesnelheid veranderen bij een toename in de substraatconcentratie.

In **hoofdstuk 6** wordt de invloed van de pH van de hydrolyse op de selectiviteit bepaald. Voor de hydrolyse van 1 % (w/v) WPI door BLP nam de algehele hydrolysesnelheid toe met een toename in pH van 7.0 naar 9.0. Omdat uit literatuur bekend is dat de stabiliteit van β-lactoglobuline afneemt met dezelfde pH toename, kan geconcludeerd worden dat de toegankelijkheid van intact β-lactoglobuline voor hydrolyse toeneemt bij een afname van de eiwitstabiliteit. Als verdere indicatie van veranderingen in het mechanisme werden peptiden geïdentificeerd en gekwantificeerd onder de verschillende condities als functie van de DH. Het bleek dat overgangs peptiden, peptiden die verder gehydrolyseerd kunnen worden, hogere concentraties bereikten bij pH 9.0 dan bij pH 7.0. De selectiviteit van het enzym voor de kniplocaties in β-lactoglobuline verandert als functie van de pH, maar op vijf verschillende manieren voor de verschillende kniplocaties. Significante veranderingen werden geobserveerd in de selectiviteit met ofwel minimumwaarden bij pH 8.0 of hoge toename en afname als functie van de pH. Ter conclusie, het veranderen van de pH van hydrolyse beïnvloed de algehele hydrolysesnelheid en het hydrolysemechanisme.

Gedurende de peptideidentificatie voor het bepalen van de enzymselectiviteit werden, op basis van de enzymspecificiteit, een aantal onverwachtte peptides geannoteerd. Het bleek dat deze a-specifieke peptides ontstonden door het knippen van eerder gevormde specifieke peptides en niet door directe hydrolyse van het intacte eiwit. Dit werd bevestigd door deze, synthetisch geproduceerde, peptiden los van elkaar te incuberen in de aanwezigheid van het enzym (**hoofdstuk 7**). Daarnaast werden dezelfde bindingen ook verbroken tijdens de incubatie van de gesynthetiseerde peptide in de afwezigheid van enzym. Dit toonde aan dat niet alle peptiden, die tijdens eiwithydrolyse gevormd worden, stabiel zijn. Deze instabiliteit wordt versterkt door de aanwezigheid van het enzym.

Tijdens de analyse van de hydrolysaten wordt een grote variatie aan peptiden gevormd. Door de parameter selectiviteit te gebruiken om de enzymwerking te kunnen karakteriseren, wordt de grote hoeveelheid gecreëerde data gereduceerd. Naast deze experimentele benadering, werd een simulatiemodel gebruikt om de eiwithydrolyse te beschrijven (**hoofdstuk 8**). Dit stochastisch model is gebaseerd op een willekeurige selectie van substraatmoleculen en kniplocaties. De invloed van het aantal kniplocaties en de verdeling van de kniplocaties in het eiwit op de selectiviteit werd aangetoond. Daarnaast werd een goede correlatie gevonden tussen de gesimuleerde en de experimentele enzymselectiviteit voor de meeste kniplocaties. Het feit dat de selectiviteit voor sommige kniplocaties niet gesimuleerd kan worden geeft aan dat het lastig is om alle parameters die de selectiviteit beïnvloeden in beschouwing te nemen.

In **hoofdstuk 9** worden de tijdens het onderzoek aangetroffen uitdagingen voor een complete beschrijving van het hydrolysemechanisme gepresenteerd. Dit omvat de obstakels in het annoteren en kwantificeren van alle aanwezige peptiden in de hydrolysaten. Daarnaast werden de ontwikkelde parameters voor het identificeren van de kwaliteit van de annotatie en kwantificatie bediscussieerd. Tenslotte werd de bepaling en toepasbaarheid van de nieuw gedefiniëerde parameter selectiviteit besproken. Met deze parameter werd duidelijk vastgesteld dat er grote verschillen bestaan tussen de hydrolysesnelheid van de verschillende kniplocaties door het enzym. De parameters, die in dit project zijn ontwikkeld voor een eenvoudig systeem, worden toepasbaar geacht om de enzymselectiviteit in complexere systemen te bepalen. Te denken valt aan mengsels van eiwitten als substraat en het gebruik van a-specifieke enzymen.

Résumé

Les hydrolysats enzymatiques de protéines sont largement utilisés dans l'industrie agro-alimentaire pour leurs qualités techno-fonctionnelles et leur valeur nutritionnelle. Afin de comprendre les différences de composition dans les hydrolysats de protéines due à des variations de conditions d'hydrolyse (par exemple, différentes enzymes ou concentrations de substrat), le mécanisme de l'hydrolyse doit être compris en détails. Les cinq paramètres généralement utilisés pour décrire l'hydrolyse et les hydrolysats sont présentés dans le **chapitre 1**. Ces paramètres sont le degré d'hydrolyse (DH), la quantité de protéine intacte restante, la distribution des masses molaires, l'identification des peptides et enfin la quantification des peptides. La plupart des études publiées utilisent seulement un ou deux de ces paramètres pour décrire l'influence des conditions d'hydrolyse et, par conséquent, ne présentent pas une analyse complète. De plus, l'identification et la quantification des peptides n'est, de manière générale, pas décrite sur l'ensemble des peptides présents dans les hydrolysats. Ceci démontre que dû à un manque d'informations cohérentes, le mécanisme de l'hydrolyse enzymatique ainsi que l'influence de la concentration en substrat et du pH ne sont que partiellement comprises.

Dans le **chapitre 2**, l'influence de la concentration initiale en protéine (1-30 % (w/v)) sur la vitesse d'hydrolyse de WPI (protéines de lactosérum) est comparée pour deux enzymes non spécifiques, Alcalase et Neutrase. Pour chaque enzyme, une augmentation de la concentration en protéine entraine une diminution de la vitesse globale d'hydrolyse pour un ratio enzyme/substrat constant, ainsi qu'une diminution du degré d'hydrolyse atteint après une incubation de deux heures. Cela confirme que l'influence de la concentration en substrat est un effet générique qui ne dépend pas de l'enzyme utilisée comme suggéré par la littérature scientifique. Afin de comprendre quels facteurs influencent l'hydrolyse, celle-ci a été conduite en présence de 0.5 M NaCl. Dans ces conditions, la conductivité de la solution de 1 % (w/v) WPI est équivalente à celle de 30 % (w/v) WPI (46 $mS.cm^{-1}$) et un DH final comparable est obtenu pour les deux solutions. Cela démontre que la force ionique de la solution est un facteur important qui influence la vitesse d'hydrolyse.

En plus de son influence sur la force ionique, l'augmentation de la concentration en substrat (jusqu'à 30 % (w/v) WPI) entraîne aussi une diminution de la quantité d'eau disponible. Cette diminution semble évidente mais n'a que rarement été considérée comme facteur d'influence sur l'hydrolyse. Afin d'évaluer la disponibilité de l'eau, l'activité aqueuse a été mesurée avant l'hydrolyse et après deux heures d'hydrolyse (**chapitre 3**). La fraction d'eau libre, calculée connaissant les valeurs d'hydratation de chaque acide aminé, est de 0.99 pour 0.1 % (w/v) WPI et de 0.83 pour 30 % (w/v) WPI. De plus, la fraction d'eau libre a aussi été déterminée en mesurant la vitesse de relaxation de l'eau par RMN. La fraction d'eau libre est alors de 0.9 pour 30 % (w/v)

WPI, ce qui confirme les valeurs obtenues par calculs théoriques. Afin d'évaluer l'excès d'eau pendant l'hydrolyse enzymatique, le ratio eau libre/eau liée a été calculé en fonction du DH. En augmentant la concentration en protéine, le ratio eau libre/eau liée décroît, ce qui est synonyme d'une diminution de la quantité d'eau disponible. La diminution de la vitesse locale d'hydrolyse avec l'augmentation de la concentration de substrat est corrélée à la diminution d'eau disponible.

Alors que la vitesse d'hydrolyse est affectée par la disponibilité de l'eau, des changements dans le mécanisme d'hydrolyse peuvent aussi être envisagés. Le grand nombre de peptides formés rend difficile la description du processus d'hydrolyse. Il est donc difficile d'identifier l'influence des conditions d'hydrolyse. Pour décrire le mécanisme d'hydrolyse, le terme *sélectivité de l'enzyme* a été introduit (**chapitre 4**). La sélectivité est définie comme la vitesse relative d'hydrolyse d'un site de clivage comparée à l'ensemble des sites de clivage dans la séquence de la protéine, pour une spécificité de l'enzyme donnée. La sélectivité a été déterminée pour l'enzyme *Bacillus licheniformis* protease (BLP), qui est spécifique pour les liaisons peptidiques après l'acide glutamique et l'acide aspartique. Pour calculer la sélectivité, les peptides obtenus à différents degrés d'hydrolyse pendant l'hydrolyse de la β-lactoglobuline ont été annotés et quantifiés. D'autres méthodes ont également été développées pour décrire la qualité de l'annotation et de la quantification des peptides. La couverture de séquence des peptides est le paramètre de qualité développé pour l'identification. Il s'agit du nombre d'acides aminés annotés sur le nombre total d'acides aminés dans les peptides annotés et les peptides manquants. La couverture de séquence molaire est le paramètre de qualité pour la quantification et indique si chaque acide aminé a été quantifié à la concentration attendue. En déterminant la sélectivité, il a été montré que la sélectivité envers chaque site de clivage de la β-lactoglobuline varie entre 0.003 % et 17 %. Cela indique de grandes différences en terme de sélectivité envers le même type de liaisons après le même type d'acide aminés. Grâce à la sélectivité, les actions de l'enzyme peuvent être décrites quantitativement et l'influence des conditions d'hydrolyse peut être étudiée.

La méthodologie et les outils développés dans le chapitre 4 ont été utilisés pour l'étude de l'influence de la concentration en substrat sur le mécanisme d'hydrolyse (**chapitre 5**). Comme décrit précédemment, la vitesse globale d'hydrolyse décroît avec l'augmentation de la concentration en substrat (0.1-10 % (w/v) WPI). L'accessibilité du substrat pour l'enzyme est la même pour toutes les concentrations en protéine. La sélectivité a été déterminée comme la vitesse relative d'hydrolyse de chaque position comparée à l'ensemble des positions possibles dans la protéine. La concentration en substrat a une influence évidente pour quatre sites de clivage, pour lesquels l'enzyme a une très haute sélectivité (± 15 %, déterminé dans le chapitre 4). À une concentration de 10 % (w/v) WPI, la sélectivité de deux de ces sites est multipliée par deux par rapport aux autres concentrations. Pour les deux autres sites, la sélectivité est divisée par deux par rapport aux autres concentrations. De plus, l'enzyme a une plus haute sélectivité envers les résidus Asp pour les concentrations en protéines élevées (1-10 %

(w/v) WPI) que pour les basses concentrations (0.1-0.5 % (w/v) WPI). Cela indique qu'à la fois la vitesse d'hydrolyse et la sélectivité de l'enzyme sont influencées par la concentration en substrat.

Dans le **chapitre 6**, l'influence du pH d'hydrolyse sur la sélectivité de l'enzyme a été déterminée. Pour l'hydrolyse de 1 % (w/v) WPI par l'enzyme BLP, la vitesse d'hydrolyse globale augmente quand le pH augmente de 7.0 à 9.0. Il a déjà été établi dans la littérature que la stabilité de la β-lactoglobuline diminue quand le pH augmente. L'accessibilité de la β-lactoglobuline intacte est par conséquent augmentée avec la diminution de la stabilité. Pour obtenir davantage d'informations sur les changements dans le mécanisme d'hydrolyse, les peptides formés ont été identifiés et quantifiés pour les différentes conditions d'hydrolyse et en fonction du DH. Les peptides intermédiaires (c'est-à-dire formés pendant l'hydrolyse mais qui peuvent encore être hydrolysés) sont davantage accumulés à pH 9.0 qu'à pH 7.0. La sélectivité présente 5 comportements différents en fonction du pH pour tous les sites de clivage de la β-lactoglobuline. Des différences importantes ont été observées avec des valeurs minimales de sélectivité obtenue à pH 8.0 ou de larges augmentations ou diminutions en fonction du pH. En conclusion, en variant le pH d'hydrolyse, non seulement la vitesse globale d'hydrolyse est changée mais aussi le mécanisme d'hydrolyse.

Lors de l'identification des peptides pour la détermination de la sélectivité de l'enzyme, un certain nombre de peptides non attendus, basé sur la spécificité de l'enzyme, ont été identifiés. Il a été déterminé que ces peptides non spécifiques sont obtenus après clivage de liaisons peptidiques dans certains peptides précédemment formés et non directement sur la protéine intacte. Cela a été confirmé en incubant ces mêmes peptides produits synthétiquement seuls en présence de l'enzyme (**chapitre 7**). De plus, en incubant les mêmes peptides seuls en l'absence d'enzyme, les mêmes clivages ont été observés bien qu'à une moindre mesure. Cela montre que les peptides formés pendant hydrolyse enzymatique ne sont pas tous stables. Cette instabilité est augmentée en présence de l'enzyme.

Lors de l'analyse des hydrolysats, une grande diversité de peptides est observée. En utilisant la sélectivité pour caractériser le mode d'action de l'enzyme, la grande quantité d'informations générée est réduite à un unique paramètre. En plus de l'approche expérimentale présentée précédemment, un modèle a été utilisé pour décrire l'hydrolyse des protéines (**chapitre 8**). Ce modèle stochastique est basé sur une sélection aléatoire du substrat puis du site de clivage. L'influence du nombre de sites de clivage possible et de leur distribution a été démontrée. De plus, une bonne corrélation a été obtenue entre les données expérimentales et le modèle. Le fait que la sélectivité pour certains sites de clivage ne soit pas modélisée correctement indique qu'il est difficile de définir l'ensemble des paramètres qui influencent la sélectivité.

Dans le **chapitre 9**, les défis rencontrés lors de l'analyse complète des hydrolysats pour définir le mécanisme d'hydrolyse sont discutés. Cela inclut les difficultés rencontrées lors de l'annotation et de la quantification de tous les peptides présents. Par ailleurs, les paramètres développés pour identifier la qualité de l'annotation et de la

quantification sont également discutés. Enfin la détermination et l'applicabilité du paramètre sélectivité sont présentées. Grâce à ce paramètre, il a clairement été établi qu'il y a de larges variations dans la vitesse d'hydrolyse envers les différents sites de clivage. Les paramètres développés pour un système simple dans ce projet peuvent être potentiellement appliqués pour déterminer la sélectivité d'enzymes dans des systèmes plus complexes, par exemple, plus de substrats ou pour des enzymes non spécifiques.

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Claire

About the author

Curriculum Vitae

Claire Isabelle Butré was born on November, 18th 1985 in Niort (France). After graduating from high school (Melle, France, 2003), she started a two year-intensive program at the National Graduate School of Chemistry of Rennes (ENSCR, 2003-2005). After this, she was admitted to the National Graduate School of Chemistry in Mulhouse (ENSCMu). In 2009, she graduated with an Engineering degree of Chemistry (Ingénieur Chimiste). She simultaneously obtained a Master degree from the University of Haute-Alsace (Mulhouse, France, 2009) in organic and bioorganic chemistry. As part of her studies, she spent one year as assistant researcher at Danisco (Brabrand, Denmark, 2007-2008), working on xanthan. She did her final internship at Laboratoires Pierre Fabre Dermo-Cosmetique as assistant researcher in organic synthesis. From April 2010 to April 2014, she was a PhD researcher in the Laboratory of Food Chemistry at Wageningen University under the supervision of Dr. Peter Wierenga and Prof. dr Harry Gruppen. The results obtained during this period are presented in this thesis.

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List of publications

C.I. Butré, P.A Wierenga, H. Gruppen, Effects of ionic strength on the enzymatic hydrolysis of diluted and concentrated whey protein isolate, *Journal of Agricultural and Food Chemistry,* **2012** 60 (22) 5644-5651.

C.I. Butré, P.A Wierenga, H. Gruppen, Influence of water availability on the enzymatic hydrolysis of proteins. *Submitted.*

C.I. Butré, S. Sforza, H. Gruppen, P.A Wierenga, Introducing enzyme selectivity: A quantitative parameter to describe enzymatic protein hydrolysis. *Accepted for publication in Analytical and Bioanalytical Chemistry* DOI: 10.1007/s00216-014-8006-2.

C.I. Butré, S. Sforza, H. Gruppen, P.A Wierenga, Influence of substrate concentration on the enzyme selectivity. *Submitted*

C.I. Butré, S. Sforza, P.A Wierenga, H. Gruppen, Influence of the pH of hydrolysis on the enzyme selectivity. *Submitted*

C.I. Butré, S. Buhler, S. Sforza, H. Gruppen, P.A Wierenga, Spontaneous, non-enzymatic, breakdown of peptides during enzymatic protein hydrolysis. *To be submitted*

P.A Wierenga, **C.I. Butré**, I.G. Stoychev, H. Gruppen, A simulation model to describe the hydrolysis of proteins by specific and non-specific proteases. *To be submitted*

Overview of completed training activities

Discipline specific activities

Courses

Enzyme kinetics and technology (DTU, LGF), Lyngby, Denmark, 2010.
Proteomics (VLAG), Wageningen, the Netherlands, 2011.
Sustainability analysis is food production (VLAG, LGF), Wageningen, the Netherlands, 2011.
Food and biorefinery enzymology (VLAG, LGF), Wageningen, the Netherlands, 2011.
Advanced food analysis (VLAG), Wageningen, the Netherlands, 2013.

Conferences and Meetings

Enzymes in sustainable food production (LGF), Halkidiki, Greece, 2013.

General courses

Project Management (LMC, LGF), Copenhagen, Denmark, 2010.
How to get success in the laboratory (LMC, LGF), Copenhagen, Denmark, 2010.
Global food production in a changing world (School of Business at Sun Yat-sen University, LGF), Guangzhou, China, 2012.
Ethics in food science (VLAG, LGF), Wageningen, the Netherlands, 2012.

Optionals

Leangreenfood project meetings and excursions, 2010-2013.
PhD trip to Switzerland and Italy, WU (FCH), 2010.
LGF trip to China, 2012.
Preparation PhD research proposal.
BSc/MSc thesis student presentations and colloquia, WU (FCH), 2010-2014.
PhD presentations, WU (FCH), 2010-2014.

Abbreviations used

LGF: Leangreenfood project

VLAG: Graduate School for nutrition, food technology, agrobiotechnology and health science.

WU: Wageningen University

FCH: Laboratory of Food Chemistry

DTU: Technical University of Denmark

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Claire Isabelle Butré, 2014