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Effects of Ionic Strength on the Enzymatic Hydrolysis of Diluted and Concentrated Whey Protein Isolate

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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 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 rate of hydrolysis for low concentrations (to 0.018 s^{-1} for 1% WPI), resulting in similar rates of hydrolysis 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.

KEYWORDS: *concentrated systems, proteins, enzymatic hydrolysis, kinetics, electrostatic interactions, degree of hydrolysis, conductivity, rate of hydrolysis, whey protein isolate*

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 eq 1.[5]

$$\frac{d(DH)}{dt} = k\frac{E}{S_0}e^{-a(DH)} \quad (1)$$

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] (eq 2) for determination of the hydrolysis parameters (k and a):

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

From eq 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

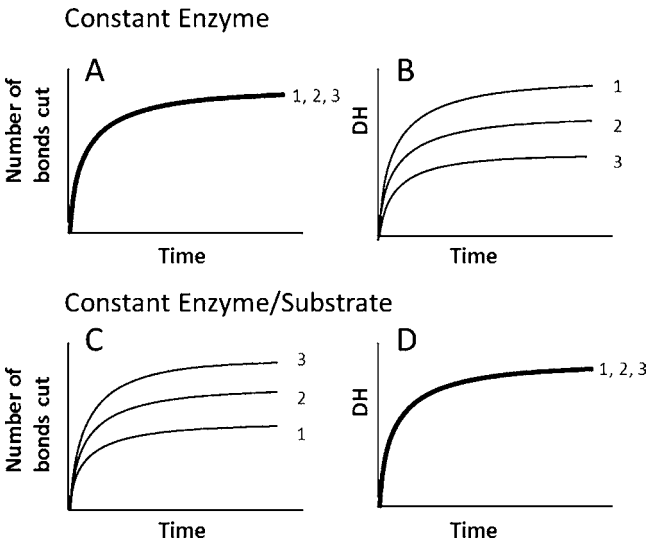

Figure 1. Overview of hydrolysis kinetics for constant enzyme concentration (A, B) and constant enzyme/substrate ratio (C, D), based on eq 1, at low (1), intermediate (2), and high (3) substrate concentration.

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Table 1. Properties of Whey Proteins

	Uniprot no.	% in Bipro	M_w	no. of amino acids	*N*-factor (g of N/g of protein)	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	na[a]	5.5	145000[b]	1218[c]	6.49[c]	176900[b]

[a]Not available. [b]From Tewari & Mukkur.[21] [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 M_w of the protein, and the N content was calculated using the average N content of the other three proteins (1.31 ± 0.03 mol of N/mol of amino acid).

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]

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 to 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 (Figure 1C,D).

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% was IGg1)[9] according to the manufacturer. The protein content of the powder was 93.4% (w/w) as determined by Dumas ($N \times 6.16$). 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 0.8 L (*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.

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 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).

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

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 (0.133 μL of enzyme/mg of 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 (0.133 μL of enzyme/mg of protein). The rate of hydrolysis was calculated from eq 2, using k_{fit} ($k_{fit} = k(E_0/S_0)a$) as the fitting parameter.

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

$$\mathrm{DH}\,(\%) = V_b \cdot N_b \cdot \frac{1}{\alpha} \cdot \frac{1}{m_p} \cdot \frac{1}{h_{tot}} \tag{4}$$

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 ($1/\alpha$ = 1.20 at 40 °C and pH 8 and $1/\alpha$ = 2.27 at 50 °C and pH 7);[10] m_p the mass of protein in g; h_{tot} = total number of peptide bonds per gram of protein substrate (8.5 mmol/g for whey protein 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 eq 5.

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

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 min, 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 × 100) at each point in time is lower than 10%.

Inactivation Test. The inactivation was verified by hydrolyzing a sample of 5% WPI for 10 min using the pH stat. The pH was afterward brought to pH 2 by addition of 5 M HCl to inactivate the enzyme. After 10 min, the pH was readjusted to 8 in the pH stat. After readjusting the pH to 8, the pH remained constant (for at least 1 h), 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*-phthaldialdehyde (OPA) on samples taken during the hydrolysis with the pH stat as described in Protein Hydrolysis. The OPA reagent was prepared as described previously.[11] Samples were diluted to 0.5% (w/v) in a 2% (w/v) SDS solution, stirred for 20 min, 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 min. The presence of alkylisoindoles 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 Fischer 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 eq 6,

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

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.[12] This conductivity was recalculated to ionic strength (equivalent concentration of NaCl) using a calibration curve (eq 7) made by measuring the conductivity of NaCl solutions of concentrations ranging from 0.01 to 0.5 M.

$$\text{ionic strength (NaCl equiv M)} = 1.02 \times 10^{-5} \times \text{conductivity } (\mu S \cdot cm^{-1}) \quad (7)$$

Size Exclusion Chromatography (SEC). 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 was 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 h 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 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., nonhydrolyzed 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 × 150 mm, 1.7 μm particle size) with an Acquity UPLC BEH C18 Vanguard precolumn (2.1 × 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 as follows: 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,[13] the retention time of peptides depends on the molecular weight of the peptides, with shorter elution times for smaller peptides. 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). 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 rate of hydrolysis with increasing substrate concentration have been described in the literature.[1,2,4]

Physical Properties and Hydrolysis Kinetics. *Solubility and Hydrolysis of the Protein 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.

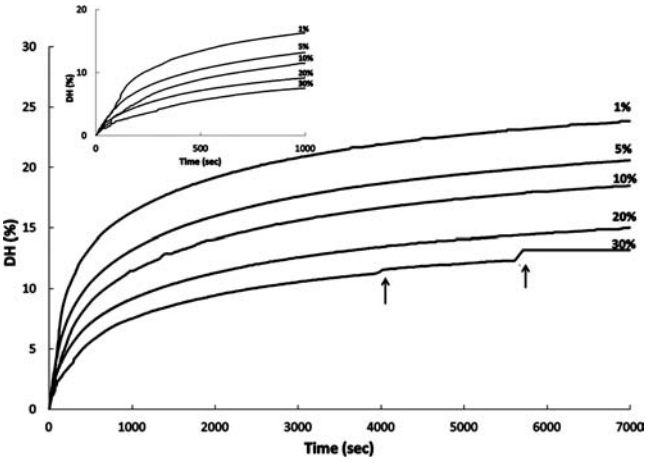

Figure 2. Hydrolysis curves of WPI suspensions hydrolyzed by Alcalase 2.4L at 40 °C for protein concentrations ranging from 1 to 30% (w/v); arrows indicate effect of gelation. Inset to show initial rate of hydrolysis.

Table 2. Properties of Whey Protein Isolate Dispersions

conc % (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

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 Materials and Methods. 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).

In the absence of nondissolved material the 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 rate of hydrolysis (k_{fit}) is calculated from the pH stat results using eq 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 rates of hydrolysis are observed for all substrate concentrations. These differences are due to the different activities of the two enzymes. For both enzymes, the rate of hydrolysis is not affected by the presence of nondissolved proteins. Furthermore, for both enzymes it is observed that the rate of hydrolysis 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.

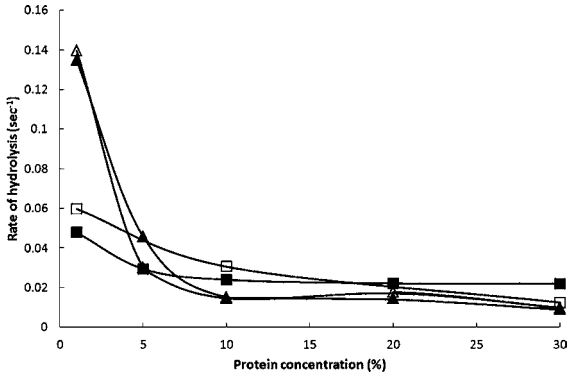

Figure 3. Rate of hydrolysis as a function of the concentration for dispersions and solutions: WPI hydrolyzed by Alcalase without removal of insoluble parts (▲) and after removal of insoluble parts (Δ); WPI hydrolyzed by Neutrase without removal of insoluble parts (■) and after removal of insoluble parts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

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).

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})	
concn % (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

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 the presence of 0.5 M NaCl. To verify if such a small increase in viscosity can explain the large effects on hydrolysis kinetics, the rate of hydrolysis was determined after increasing the viscosity of a 1% (w/v) WPI solution by addition of either glycerol (up to 70%) or locust bean gum (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 s (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 rate of hydrolysis, 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

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concentrations does not explain the observed effect of slower rate of hydrolysis 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 $mS{\cdot}cm^{-1}$ 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.[12] 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.[14] 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

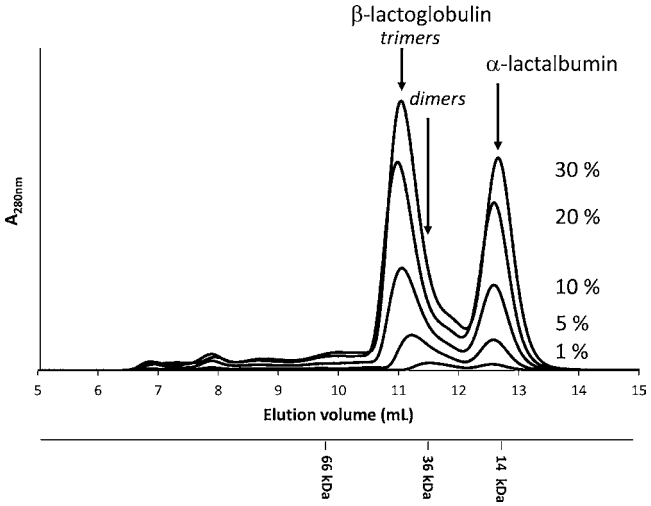

Figure 4. SEC chromatograms of intact WPI injected at different concentrations (1−30% (w/v), sample and elution buffer contain 10 mM phosphate pH 8 and 0.5 M NaCl).

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).

For 1−30% (w/v) protein solutions (Figure 5A), the rates of hydrolysis by Alcalase in the absence and presence of 0.1 and 0.5 M NaCl were determined. The curves for protein concentrations >10% (w/v) are slightly affected by the increased ionic strength; the rate of hydrolysis (as determined from eq 2) is slightly increasing with increasing ionic strength at

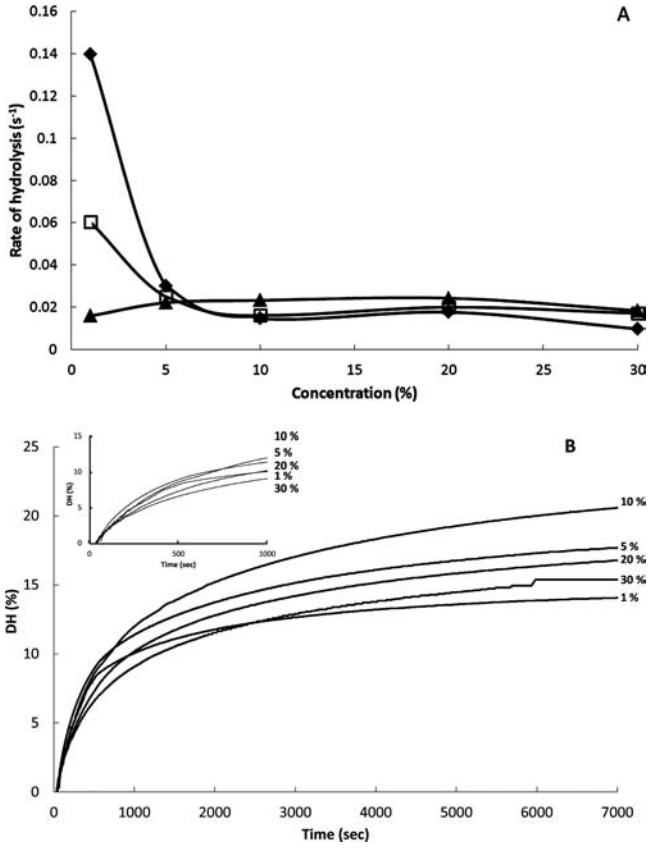

Figure 5. (A) Rates of hydrolysis of WPI solutions hydrolyzed by Alcalase (at 40 °C) in presence of 0 M NaCl (◆), 0.1 M NaCl (□), 0.5 M NaCl (▲). (B) Hydrolysis curves of WPI solutions hydrolyzed by Alcalase 2.4L at 40 °C for protein concentrations ranging from 1 to 30 % (w/v) in presence of 0.5 M NaCl.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

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). 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.[15] There, a five times decrease of the rate of hydrolysis 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 eq 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 to 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 rate of hydrolysis and in the final DH. While the decrease in hydrolysis rate is similar in both cases, the conductivity is not (44 $mS \cdot cm^{-1}$ 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 rate of hydrolysis, 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 the 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. 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.

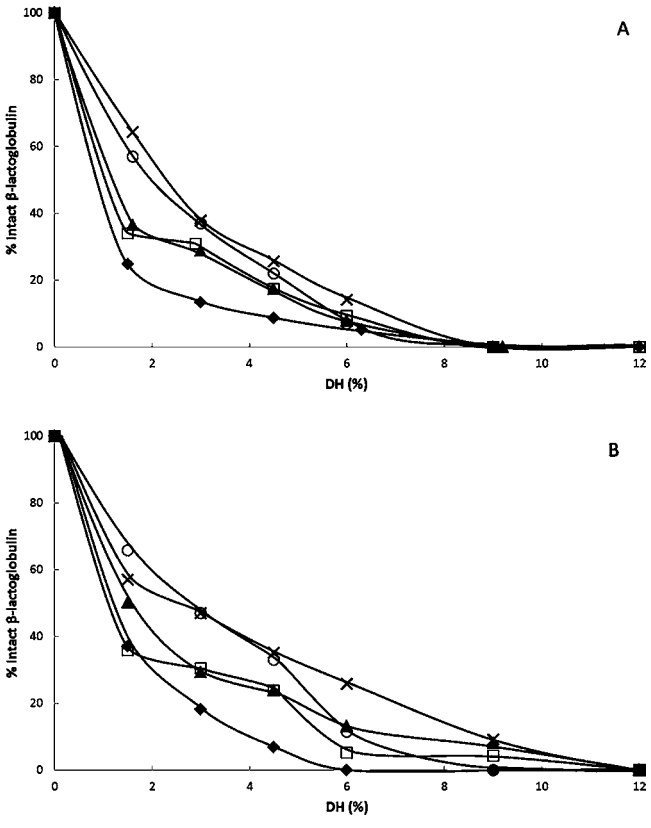

Figure 6. Proportion of intact β-lactoglobulin as a function of the degree of hydrolysis at different initial protein concentrations: (A) for the protein solutions; (B) for the protein solutions in 0.5 M NaCl. At different initial protein concentrations: (◆) 1%, (□) 5%, (▲) 10%, (×) 20%, (○) 30%.

Peptide Profile. RP-HPLC 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

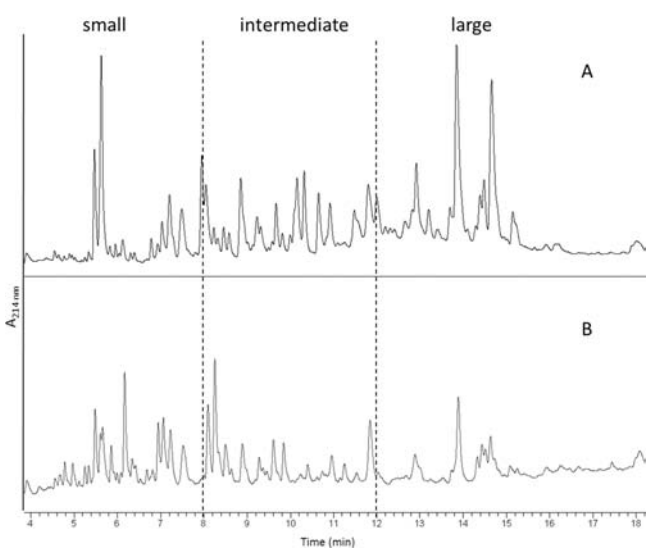

Figure 7. RP-HPLC chromatograms of the hydrolysates of 1% initial protein concentration at DH = 9% at 0 M NaCl (A) and 0.5 M NaCl (B).

was determined. 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).[16] 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

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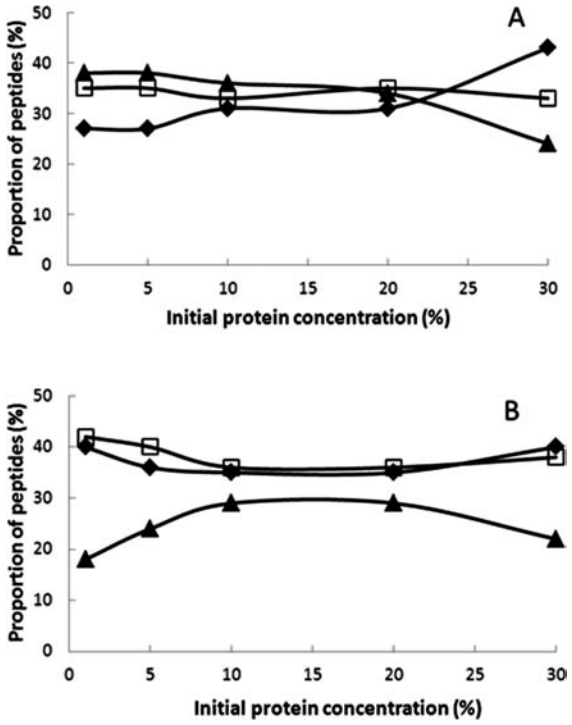

Figure 8. Proportion of (◆) small peptides, (□) intermediate peptides, and (▲) large peptides in the hydrolysates as a function of the initial protein concentration at DH = 9% at 0 M NaCl (A) and 0.5 M NaCl (B).

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.[13]

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,[15] but was not linked in later studies to the observed decrease with increasing substrate concentration. It was proposed that this decreased rate of hydrolysis with increasing ionic strength could be due to increased structural stability of the protein.[15] The increase of structural stability at higher ionic strength has indeed been shown for β-lactoglobulin,[17] and no significant effect was observed for whey protein isolate (WPI).[18] 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.[17] The thermal stabilities with increasing WPI concentration from 2% to 10% were found to be similar,[18] while the hydrolysis rates are clearly not (Figure 5A). This shows that the differences in 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,[19] but resulted in an increased rate of hydrolysis of synthetic peptide substrates by thermolysin.[20]

It has been shown that the hydrolysis of synthetic peptides by Alcalase is not affected by NaCl concentrations from 0 to 0.9 M NaCl.[19] Therefore, it is concluded that the decreased rate of hydrolysis 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,[18] 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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Notes

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