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Influence of salts on hydrolysis of β-lactoglobulin by free and immobilised trypsin

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ABSTRACT

Immobilised trypsin is an alternative to free trypsin for producing protein hydrolysates with increased functionalities. However, the influence of hydrolytic conditions on this process remains unclear. The influence of salts on β-lactoglobulin (β-Lg) hydrolysis by free and immobilised trypsin was compared. For both forms of trypsin, 0.1 M Tris accelerated the release of most final peptides except f (71–75), and had no significant effects on the hydrolysis of intact β-Lg. Increasing NaCl concentrations from 0 to 0.02 M increased the degree of hydrolysis (DH) by 22.4% for free trypsin versus 62.1% for immobilised trypsin. The presence of 0.1 or 0.5 M NaCl hindered the release of peptides associated with the breakdown of intact protein. This led to 2–4 fold decreases in depleting intact β-Lg and DH, except immobilised trypsin at 0.1 M NaCl (DH increased by 44.3% versus without NaCl). Potential mechanisms underlying the effects of salts are discussed.

1. Introduction

In the past several decades, bovine β-lactoglobulin (β-Lg), a major allergen absent from human milk, has been subject to extensive investigations regarding enzymatic processing via various enzymes (Hernández-Ledesma, Recio, & Amigo, 2008). Trypsin (EC 3.4.21.4), a serine protease, is found in the digestive system of humans and many other vertebrates. Tryptic hydrolysis of β-Lg results in reduced allergenicity (Selo et al., 1999) and enhanced nutritional values (Hernández-Ledesma et al., 2008; Leeb, Gotz, Letzel, Cheison, & Kulozik, 2015). However, the costly production and purification of trypsin seriously limits its implementation in the food industry (Yu & Ahmedna, 2012). As an alternative approach, immobilisation of trypsin has attracted more and more attention due to the possibility of reusing the enzyme and producing enzyme-free hydrolysates.

In our previous work (Mao, Černigoj, Zalokar, Štrancar, & Kulozik, 2017), a monolithic column-based immobilised trypsin reactor (MITR) showed significant activity toward β-Lg. The immobilisation of an enzyme can alter its intrinsic properties (V_{max}, k_{cat} or K_m) (Duggal & Bucholz, 1982), leading to changes in enzyme selectivity, i.e., the rate at which individual cleavage sites in a protein substrate are hydrolysed relative to other cleavage sites (Butré, Sforza, Gruppen, & Wierenga, 2014). Consistent with these observations, we compared the selectivity of free and immobilised trypsin for β-Lg hydrolysis focusing on the impact of hydrolytic pH, and found that pH had a greater effect on the selectivity of free trypsin compared with immobilised trypsin (Mao, Krischke, Hengst, & Kulozik, 2018). Except for pH, the influence of salts on hydrolysis process is also important, because the production of food protein hydrolysates is never conducted in a pure aqueous media, but rather in a complex system with various ions.

Salt-effect studies have proven useful for determining the intrinsic properties of enzymes (Endo, Kurinomaru, & Shiraki, 2016, 2018; Garajova et al., 2017; Quan et al., 2008; Salis, Bilanicova, Ninham, & Monduzzi, 2007). Most of these studies interpret the salt-effect data based on ionic properties and ion specificity. Ionic properties refer to the effects of any salt ion, including charge shielding/electrical double layer effects and stoichiometric ion binding to a charged protein (Tsumoto, Ejima, Senczuk, Kita, & Arakawa, 2007). These effects are always independent on the salt type, but dependent on the salt concentration (ionic strength). Ionic properties are important in regulating enzyme–substrate interactions, thereby affecting enzyme activity and selectivity. This factor is particularly important in hydrolysis reactions that depend

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on the movement of charged molecules relative to each other. In tryptic hydrolysis. Asp_{189}, located in the catalytic pocket of trypsin, attracts and stabilises positively charged Lys and Arg residues on substrates (Evnin, Vásquez, & Craik, 1990). Thus, both the binding of charged substrates to the enzyme and then the movement of charged groups within the catalytic active site will be influenced by the ionic composition of medium (Chaplin & Bucke, 1990).

The ion-specific effect of medium on proteins was first reported in a systematic way by Franz Hofmeister (Hofmeister, 1888), who ranked the ability of ions at a fixed ionic strength to affect the properties of proteins in aqueous solutions. Hofmeister differentiated between chaotropes and kosmotropes, salts that induced either disorder or more order, respectively, in protein conformation. Hofmeister ions are reported to influence the properties of numerous enzymes (Endo et al., 2016; Garajova et al., 2017; Tougu et al., 1994). Mostly, the ion-specific influence on enzyme activity follows the Hofmeister series, with kosmotropes activating enzymes and chaotropes inhibiting enzyme activity (Garajova et al., 2017). Enzyme activation by kosmotropes occurs because these salts increase both the structural stability of the enzyme and the hydrophobic interactions between the enzyme and its substrate (Endo et al., 2016). A bell-shaped dependence of enzyme activity on ions in the Hofmeister series has been observed, indicating that both chaotropic and kosmotropic ions can inactivate enzymes (Žoldák, Sprinzl, & Sedlák, 2004).

Chymotrypsin exhibits increased activity with the addition of 3 M NaCl, but no significant increase in the presence of 3 M LiCl or KCl (Wesolowska, Krokoszynska, Krowarsch, & Otlewski, 2001). Another study observed that with the addition of 0.5 M NaCl, the K_m of chymotrypsin decreased and its K_{cat} increased, while trypsin showed the opposite results (Endo, Kurinomaru, & Shiraki, 2018). In addition, weak electrolytes are widely applied as buffers in enzyme technology. Tris (hydroxymethyl) aminomethane (Tris) solution is one such buffer, which is reported to stabilise BSA molecules (Taha & Lee, 2010) and interact with lysozyme molecules through hydrogen-bonding (Quan et al., 2008).

Previous studies regarding salt effects on enzymes have used primarily low–molecular-mass substrates (Endo et al., 2018, 2016; Tougu et al., 1994). These results may not apply to enzymatic reactions involving proteins as substrates, e.g., β-Lg in this study, as salts may influence β-Lg structure, thus affecting the enzymatic process. For instance, Renard, Lefebvre, Griffin, and Griffin (1998) found that β-Lg favoured dimerisation in the presence of NaCl. Furthermore, ions also affect the tertiary structure of β-Lg, e.g., Trp exposure increases in the presence of NaCl (>0.1 M), indicating that more hydrophobic groups are exposed (Zhao, Li, & Li, 2017). Therefore, the influence of salts on the enzymatic hydrolysis of a specific substrate should be investigated systematically on a case-by-case basis.

There appear to be no studies dealing with the influence of salts on the protein hydrolysis in a flow-through reactor using immobilised trypsin. In the present study, trypsin was covalently immobilised on an aldehyde-activated monolith through multiple attachment points. This covalent immobilisation is the most common approach to stabilise the enzyme against different denaturing conditions (Mozhaev, Melik-nubarov, Sergeeva, Šikšnis, & Martinek, 1990). Hence, compared with free trypsin, the ion-induced conformational effects on immobilised trypsin, in theory, should be significantly reduced. Additionally, substrates are convectively transported to immobilised trypsin in a flow-through MITR. This critical step is largely decided by the mass transfer properties in MITR. It is reasonable to assume that the mass transfer properties in MITR might change with the addition of salts, due to their effects at least on charge-based attraction or repulsion between substrate molecules and the monolith surface.

Therefore, the aim of this study was to determine the influence of salts on immobilised trypsin for β-Lg hydrolysis, and simultaneously to compare with free trypsin. Specifically, the following parameters were investigated: (i) ion-specific effects, i.e., buffer salt (weak organic electrolyte, Tris) versus neutral salt (strong inorganic electrolyte, NaCl); and (ii) ionic strength effects (ionic properties) using different salt concentrations. The influence of these factors was evaluated by measuring the hydrolysis efficiency and analysing the hydrolysates profiles.

2. Materials and methods

2.1. Materials

Bovine β-Lg was fractionated from whey protein isolate (WPI), a product developed by Fonterra Co-operative Group Ltd (Auckland, New Zealand), as described by Toro-Sierra, Tolkach, and Kulozik (2011). The β-Lg powder obtained had a protein content of 98.6% relative to the dry matter. β-Lg powder was dissolved in deionised water at 4 °C overnight. After the removal of denatured β-Lg by adjusting the pH to 4.6 and centrifugation (6000 ×*g* for 10 min), the supernatant was filtered through a cellulose membrane with a cut-off of 0.45 μm (Macherey–Nagel, Düren, Germany). After the pH adjustment to 8.7, β-Lg solution was mixed with pre-dissolved NaCl or Tris buffer solution to reach a final concentration of 10 mg mL^{-1} native protein, and the final pH of the mixed solutions was adjusted to 8.7.

Trypsin from bovine pancreas (Type I, approximately 10,000 Nα-benzoyl-L-arginine ethyl ester [BAEE] units mg^{-1} protein), BAEE (B4500), Tris (hydroxymethyl)-aminomethane (Tris), NaCl, and NaOH were purchased from Sigma Aldrich (St Louis, MO, USA). Deionised water was acquired using the Milli-Q System (Millipore Corporation, Bedford, MA, USA).

An aldehyde (ALD) activated CIMmultus™ column (1 mL-bed volume, BIA Separations, Ajdovščina, Slovenia)-based MITR with 2.15 ± 0.1 μm pore size was developed in our earlier work (Mao et al., 2017). The amount of immobilised trypsin was 5.0 ± 0.2 mg per MITR, and the MITR permeability was approximately 2.45×10^{-12} m^2 using deionised water.

2.2. Trypsin activity toward BAEE substrate

The enzymatic activity of trypsin was measured at pH 8.7 using the model substrate BAEE in three buffers, 0.1 M Tris buffer, 0.1 M Tris + 0.1 M NaCl, 0.5 M Tris buffer. The measurement was a spectrophotometric determination as described previously (Mao et al., 2017). The activity of free trypsin is expressed as BAEE units mg^{-1}. One BAEE unit produces a ΔA_{253} of 0.001 per min in a reaction volume of 3.20 mL. The activity of MITR was calculated as the unit U* (μmol min^{-1}), which is the amount of BAEE converted to BA by the MITR in 1 min. U* can be easily converted to BAEE units, using the conversion factor 270, as determined by Bergmeyer (1974).

2.3. Hydrolysis of β-Lg

A previous study (Cheison, Leeb, Toro-Sierra, & Kulozik, 2011b) indicated that at the optimal temperature of free trypsin, i.e., 37 °C, the enzyme selectivity was little controlled by other environmental conditions, as temperature was the dominating influence. It is expected to regulate the trypsin selectivity with the addition of salts. On the other hand, both free trypsin and MITR already showed significant activity toward β-Lg at ambient temperature (Mao et al., 2017). Hence, all hydrolysis experiments were conducted at 25 ± 1 °C.

To assess the effects of salts, 25 mL β-Lg solution (native protein, 10 mg mL^{-1}) in Tris buffer (0.1 or 0.5 M) or Tris-NaCl buffer (0.1 M Tris + 0.1 M NaCl) was hydrolysed by free trypsin or immobilised trypsin for 1 h. The hydrolysis of β-Lg by free trypsin was conducted at an enzyme/substrate ratio of 0.1% (w/w). Thus, the hydrolysis reaction was ensured to be performed at similar ratios of activity units (BAEE units) to per gram β-Lg for both free trypsin and MITR.

To further explore the effects of salts, 100-mL β-Lg solution (10 mg mL^{-1}) in 0.1 M Tris, or in 0, 0.02, 0.1, or 0.5 M NaCl was hydrolysed by free trypsin or immobilised trypsin for 3 h. During hydrolysis, 1-mL samples were taken out at intervals (0, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min). A TitroLine alpha plus auto-titrator (Schott AG, Mainz, Germany) was used to maintain a constant pH of 8.7 throughout the course of the reaction. The degree of hydrolysis (DH) was calculated according the amount of NaOH consumed, as described previously (Mao et al., 2017). In addition, because 18 of the 161 peptide bonds of β-Lg are potential cleavage sites for trypsin, a theoretical DH_{max} of 11.18% can be achieved during trypsinolysis. The practical DH_{max} values were reached and discussed in our previous work (Mao et al., 2017). In this study, all hydrolysis processes were stopped at intermediate stages to save time. Also, it makes more sense to focus on the release of peptides at the early stage of hydrolysis to explore the enzymatic selectivity.

For the hydrolysis by free trypsin, a constant enzyme–substrate (E/S) ratio of 0.1% (w/w) was applied, and the hydrolysis of 1-mL aliquots was stopped by the addition of 0.5 mL trypsin inhibitor solution (10 mg mL^{-1}, from chicken egg white, Sigma Aldrich). Hydrolysis by MITR was realised in a flow-through system (Äkta explorer, GE Healthcare Europe GmbH, Freiburg, Germany) controlled by Unicorn Software 5.31.

2.4. Analysis of hydrolysates

Hydrolysates profiles were quantitatively analysed for residual native protein content and peptide composition.

2.4.1. Quantification of residual native β-Lg

The native β-Lg content of samples was determined via reversed-phase high-performance liquid chromatography (RP-HPLC) using an Agilent 1100 series HPLC system (Agilent Technologies, CA, USA) and a PLRP-S-300 Å-8 μm column (150 × 4.6 mm, Latek, Eppelheim, Germany). Each sample was diluted with deionised water to ≈4 mg mL^{-1}. After adjusting the pH to 4.6, the sample was filtered through a cellulose membrane with a cut-off of 0.45 μm and 20 μL was injected. Detailed gradient information was previously described by Leeb et al. (2015). The protein concentration was calculated from the detected peak area and a calibration curve using standards β-Lg A (99% purity, Sigma Aldrich) and β-Lg B (99% purity, Sigma Aldrich).

2.4.2. Chromatographic separation and quantification of peptides

All samples were diluted to approximately 4 mg mL^{-1}, and 1-mL aliquots of the diluted solution were firstly incubated with 150 μL 80 mM dithiothreitol (DTT) at pH 8 and 37 °C for 45 min, and then mixed with 200 μL 400 mM chloroacetamide (CAA) and stored in the dark for 30 min. The pre-treated samples were analysed on the Agilent 1100 series HPLC system coupled with a Kinetex_XB-C18-100 Å column (100 × 4.6 mm, Phenomenex, Torrence, CA, USA). The mobile phase of solvent A containing 0.1% (v/v) trifluoroacetic acid (TFA) dissolved in Milli-Q water and solvent B consisting of 0.0555% (v/v) TFA dissolved in 80% (v/v) acetonitrile (diluted in Milli-Q water) was applied. The entire analysis was conducted at 60 °C and 1.5 mL min^{-1}. The gradient was increased multi-linearly from 1 to 45% of solvent B in 60 min. The sample injection volume was 60 μL, and the elution was monitored at 214 nm. The samples with the highest DH at each condition were further fractionated. Peaks eluted before 45 min were automatically collected separately based on the slope, and peaks after 45 min were collected based on time with a unit of 1 min. The collected fractions were analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) to determine the peptide composition.

The molar concentration of each specific peptide was calculated from the peak area according to Eq. (1) (Fernández & Riera, 2013) as follows:

$$X_i = 1 \times 10^6 \left(\frac{A_i}{\varepsilon_i \times l \times v} \right) \times f \times D \quad (1)$$

where x_i (μM) is the concentration of peptide i, Ai (AU min) is the peak area, l (0.6 cm) is the path length of the UV cell, v is the injection volume (60 μL), f is the flow rate (1.5 mL min^{-1}), D is the dilution factor of the sample before injection, and ε_i (AU M^{-1} cm^{-1}) is the molar extinction coefficient of peptide i at 214 nm, as calculated according to Kuipers and Gruppen (2007). To quantitatively compare the released peptides, the following indexes were calculated:

$$R_1(\%) = \frac{\textit{The amount of released peptide}(\mu m)}{\textit{The amount of hydrolyzed protein}(\mu m)} \quad (2)$$

$$R_2(\%) = \frac{\textit{The relative amount of released peptide}\ (\%)}{\textit{The relative DH}(\%)} = \frac{\dfrac{\textit{The amount of the released peptide}(\mu m)}{\textit{The theoretical maximun amount of each peptide}(\mu m)}}{\dfrac{\textit{The reached DH Value}(\%)}{\textit{The theoretical maximum DH value}(\%)}} \quad (3)$$

2.4.3. MALDI-TOF-MS

The fractions obtained were analysed for mass composition using a MALDI-TOF-MS system (ultrafleXtreme MALDI-TOF-TOF, Bruker Daltonics GmbH, Bremen, Germany). Matrix α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxyacetophenone (DHAP) were used separately. HCCA is sufficient to measure peptides and proteins in the low mass range from 500 to 4000 Da, and DHAP is commonly used for the high mass range up to 20,000 Da. One microlitre samples (including blank sample) or standards (PAS with HCCA and Protein Calibration Standard I with DHAP, Bruker Daltonics GmbH) were mixed with 1-μL matrices directly on the anchor target (stainless steel MTP 384, Bruker Daltonics GmbH). Each sample was spotted at least three times. The MALDI-TOF-MS was run in a positive reflection mode for mass ranges of 400–4000 Da (sample with HCCA) and 3000–10,000 Da (sample with DHAP), or in a positive linear model for the mass range of 4000–20,000 Da (sample with DHAP). The process was managed using flexControl™ 3.0 Software (Bruker Daltonics GmbH). Peptides were identified by comparing the detected mass/charge (m/z) with the theoretical m/z as described previously (Mao et al., 2017).

2.5. Statistical analysis

All experiments were performed in triplicate and in a staggered manner (for MITR) to reduce bias. Mean values ± standard deviation are reported. Analysis of variance was performed to estimate differences between mean values where the significance level was established as $P < 0.05$. The Tukey-test was used to evaluate the significance of differences. Data were plotted using Origin Pro 9.0 or R 3.3.3 (open-source software).

3. Results and discussion

3.1. General comparison of salt effects on free and immobilised trypsin

The conductivities of the applied buffers were 3.82 mS cm^{-1} (0.1 M Tris), 10.85 mS cm^{-1} (0.1 M Tris + 0.1 M NaCl), and 9.61 mS cm^{-1} (0.5 M Tris), respectively. The enzymatic activity toward BAEE (342.82 Da) and the depletion of intact β-Lg in these buffers were investigated. In all reactions, applied buffers kept pH in 8.6–8.7.

As shown in Fig. 1, either the addition of 0.1 M NaCl or increasing Tris concentration from 0.1 to 0.5 M did not significantly affect the enzymatic activity of free and immobilised trypsin, although the conductivity of the reaction medium increased greatly. Wesolowska et al. (2001) found that the activity of free trypsin toward BAEE was nearly unaffected by the presence of NaCl up to 3 M. Contrastively, the amount of hydrolysed intact β-Lg decreased significantly for both forms of trypsin due to the addition of salts, as shown in Fig. 2. For free trypsin, around 63% of intact protein was hydrolysed in 0.1 M Tris, which decreased by 40% with the addition of 0.1 M NaCl. For immobilised trypsin, the hydrolysed amount of intact β-Lg decreased by 18% with the presence of 0.1 M NaCl or 0.5 M Tris.

The surface charge of β-Lg is much more complex than BAEE. Thus, the influence of salts on electrostatic interactions between substrate and enzyme appears to be greatly strengthened, when β-Lg substituted the substrate BAEE. In addition, salts may stabilise or destabilise β-Lg. For instance, Tris buffer is reported to stabilise macromolecules such as BSA (Taha & Lee, 2010). Increasing concentrations of NaCl (up to 2 M) are reported to increase the thermal stability of β-Lg (Vardhanabhuti & Foegeding, 2008). Stabilisation of substrate molecules may prevent them from hydrolysis.

Although Tris and NaCl did not affect enzymatic activity significantly, they exerted considerable influences on the interactions between β-Lg and trypsin. To interpret the mechanism, Tris and NaCl were introduced to the reaction medium separately, and the hydrolysis without the addition of salts was conducted as well.

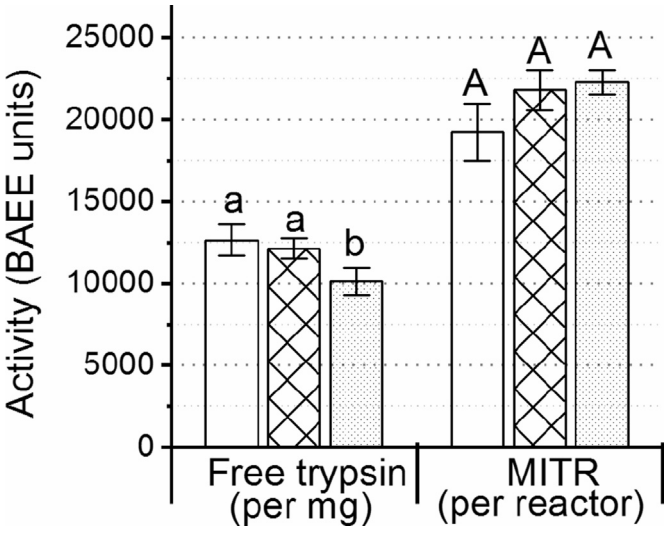

Fig. 1. Effects of Tris buffer and NaCl on trypsin activity. Hollow bars, bars with crossed lines and dots represent the hydrolysis of β-Lg in 0.1 M Tris, 0.1 M Tris + 0.1 M NaCl, and 0.5 M Tris, respectively. Data values represent mean values of three replicates with a 95% confidence interval. Values with the same lowercase letter (for free trypsin) or with the same uppercase letter (for monolith-based immobilised trypsin reactor) are not statistically different ($P \geq 0.05$).

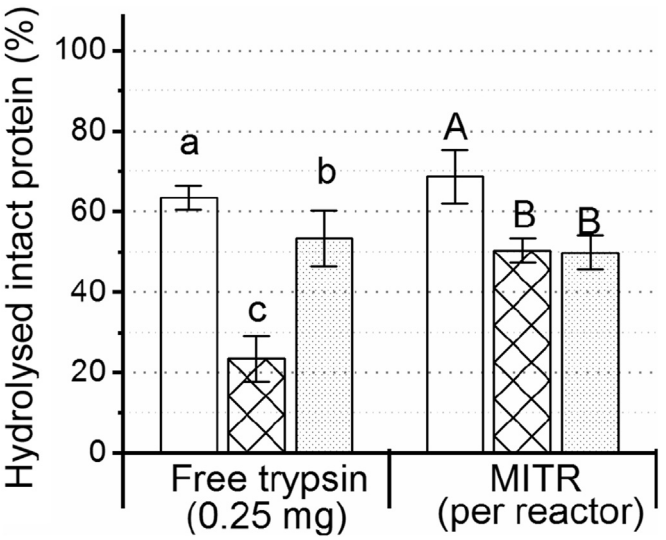

Fig. 2. Effects of Tris buffer and NaCl on the hydrolysis of intact β-lactoglobulin. Hollow bars, bars with crossed lines and dots represent the hydrolysis of β-Lg in 0.1 M Tris, 0.1 M Tris + 0.1 M NaCl, and 0.5 M Tris, respectively. Data values represent mean values of three replicates with a 95% confidence interval. Values with the same lowercase letter (for free trypsin) or with the same uppercase letter (for monolith-based immobilised trypsin reactor) are not statistically different ($P \geq 0.05$).

3.2. Influence of Tris buffer

Both 10 mg mL^{-1} β-Lg in 0 (1.05 mS cm^{-1}) and 0.1 M (3.82 mS cm^{-1}) Tris buffers were hydrolysed by free trypsin or MITR for 3 h. Because of the buffering capacity of Tris, the DH measured by a pH drop does not represent the actual extent of the reaction. Thus, the amount of intact protein hydrolysed was used to follow the reaction progress. For free trypsin, 65.7% (without Tris) and 63.5% (with Tris) of the β-Lg were hydrolysed after 1 h, while MITR required 3 h to hydrolyse similar amounts of β-Lg (64.4% without Tris; 66.6% with Tris). The peptide profiles of these four samples were analysed by HPLC and MALDI-TOF-MS.

3.2.1. The effects of Tris on peptide profiles

As shown in Fig. 3, peaks which eluted before 45 min contained peptides with a molecular weight below 3000 Da. Most of these peaks were assigned to final peptides based on trypsin specificity (as shown in Table 1), except the cleavage at Tyr_{20}–Ser_{21}, which is a nonspecific (chymotrypsin-like) cleavage by trypsin, as reported in other studies (Butré, Sforza, Wierenga, & Gruppen, 2015; Cheison, Leeb, Letzel, & Kulozik, 2011a). Although the HPLC profiles of these four samples before 45 min were quite similar, the amounts of individual peptides (mainly the final peptides) differed significantly. As illustrated in Fig. 4, the relative amount of each peptide based on the hydrolysed protein content (value R_1) is compared, and the theoretically maximum R_1 value should be 100%. The effects of Tris on the release of peptides, as indicated by R_1 values, are summarised in Table 2. Compared with hydrolysis in water, the presence of 0.1 M Tris contributed to the release of most final peptides for both forms of trypsin, except f (71–75). This peptide is typically released quickly during tryptic hydrolysis because of its external position in the three-dimensional structure of β-Lg (Fernández, Suárez, Zhu, FitzGerald, & Riera, 2013). However, its precursor peptide f (70–75) accumulated significantly in Tris buffer, indicating that its further hydrolysis was prevented. Other intermediate peptides f (41–60), f (92–101) and f (76–91) also accumulated more in 0.1 M Tris than in water for both forms of trypsin, e.g., 6-fold (for free trypsin) and 7-fold (for MITR) increases

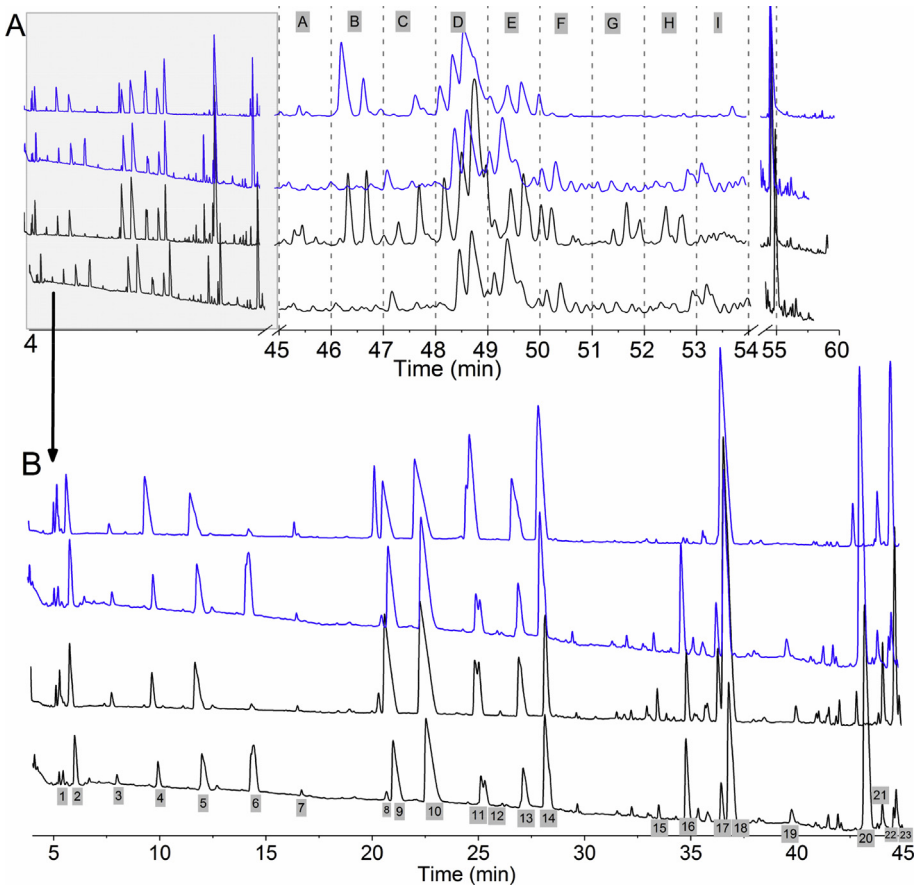

Fig. 3. HPLC profiles of β-lactoglobulin hydrolysates generated from free or immobilized trypsin in the presence or absence of Tris buffer. The peaks eluted after 45 min are illustrated in A, and the peaks eluted before 45 min are highlighted in B. Samples from top to bottom are hydrolysates produced in 0.1 M Tris by immobilised trypsin, in water by immobilised trypsin, in 0.1 M Tris by free trypsin and in water by free trypsin, respectively. Those peaks marked with number or letter are identified and reported in Table 1.

in f (41–60) were observed. The peptide f (41–60) possesses one missed cleavage site Lys_{47}, which links to a proline residue and is resistant to trypsinolysis, as observed by Olsen, Ong, and Mann (2004). Particularly for MITR, the amount of f (125–138) increased greatly. Regarding peptides eluted after 45 min, peaks representing f (102–138)b and f (15–141)a showed increasing area in the presence of Tris for both forms of trypsin.

3.2.2. The mechanism underlying the observed influence of Tris

Compared with the hydrolysis without Tris, the addition of 0.1 M Tris did not reduce the hydrolysis of intact β-Lg, while contributed to the release of most final peptides. Taha and Lee (2010) reported that Tris preferentially interacted with the peptide backbone by virtue of its −OH and amine groups through hydrogen bonding. Quan et al. (2008) found that Tris molecules formed hydrogen bonds with Asp_{52}, Glu_{35}, and Ala_{107} residues in lysozyme. Hence, it is speculated that Tris molecules mainly interact with certain intermediate peptides (the precursors of final peptides) and trypsin molecules through hydrogen bonds simultaneously, enhancing their interactions and then improving their hydrolysis. To determine whether Tris molecules preferentially interact with certain polypeptides, the characteristics of intermediate peptides are summarised in Supplementary material Table S1, including isoelectric point (PI), hydrophilicity, and the ratio of hydrophilic residues to total number of residues (%). However, there is no clear correlation between the influence of Tris and the peptide characteristics.

3.3. Influence of NaCl

Samples with 10 mg mL^{-1} β-Lg in 0 (1.05 mS cm^{-1}), 0.02 (3.25 mS cm^{-1}), 0.1 (10.25 mS cm^{-1}) and 0.5 M (46.55 mS cm^{-1}) NaCl were hydrolysed by free trypsin or MITR for 3 h.

3.3.1. The effects of NaCl on DH and amounts of residual intact β-Lg

The DH as a function of time is presented in Fig. 5A1 for free trypsin and in Fig. 5B1 for immobilised trypsin. The hydrolysis of β-Lg in water by free trypsin reached a DH of 5.87 ± 0.79% after 3 h, which was significantly higher than that by MITR (3.93 ± 0.74%). In both cases, the fastest increase in DH was observed with the addition of 0.02 M NaCl, where DH values reached 7.18 ± 0.76% (free trypsin) and 6.37 ± 0.95% (MITR), respectively. Interestingly, increasing NaCl concentration from 0 to 0.1 M significantly promoted hydrolysis efficiency by MITR, reaching DH 5.67 ± 0.75%, but it decreased DH from 5.87 ± 0.79% to 5.14 ± 0.82% for free trypsin. The addition of 0.5 M NaCl seriously hindered the hydrolysis, irrespective free or immobilised trypsin applied, i.e., the DH values were only 3.83 ± 0.82% and 2.74 ± 0.81%, respectively.

Regarding the hydrolysis of intact protein, the Linderstrøm-Lang theory presents two models: “zipper” and “one-by-one” (Adler-Nissen, 1976). In a “one-by-one” model, intact protein will slowly break down and no appreciable amounts of intermediate peptides will be accumulated, while a much faster degradation of intact protein at the initial stage of hydrolysis will be observed in a “zipper” model. In fact, most proteins show an intermediate

Table 1
Identification of peptides generated from the hydrolysis of β-lactoglobulin (β-Lg) by free and immobilised trypsin.

Peaks	Calculated mass[a]	Observed mass[b]	Assigned sequence[c]
1	1180.2	1180.5	f (61–69)a
	1122.2	1122.5	f (61–69)b
	1308.4	1307.4	f (61–70)a
	1250.4	1249.3	f (61–70)b
2	595.4 ^{Na}	595.4	f (71–75)
3	739.4^{K}	739.3	f (70–75)
4	938.5	938.5	f (84–91)
5	695.4 ^{Na}	695.4	f (9–14)
6	825.0 ^{Na}	825.0	f (71–77)
7	1245.6	1245.8	f (125–135)
8	696.8	696.9	f (15–20)
9	955.5 ^{Na}	955.5	f (1–8)
10	837.5	837.5	f (142–148)
11	1194.4	1193.5	f (92–101)
12	674.4	674.5	f (78–83)
13	904.1	904.1	f (76–83)
14	1658.7 ^{Na}	1659.0	f (125–138)
15	2092.4	2092.3	f (84–101)
16	1949.1	1949.3	f (125–141)
17	1802.1	1802.2	f (76–91)
18	1754.893^{K}	1753.6	f (149–162)
19	/	2189.1	/
20	/	1658.0/3315.1	/
21	2069.3^{k}	2069.2	f (21–40)
22	2313.3	2313.2	f (41–60)
23	2820.2	2820.2	f (102–124)b
	2848.3	2848.2	f (102–124)a
A	4075.6	4075.8	f (102–135)b
	4203.7	4203.5	f (101–135)a
	4213.7^{K}	4215.2	f (101–135)b
B	4437.9	4437.0	f (102–138)b
	14480.5	7240.1 *	f (15–141)b
C	3546.0	3544.4	f (41–70)b
	7195.2	3597.8 *	f (78–138)a
	7167.1	7167.9	f (78–138)b
D	3626.0 ^{Na}	3624.7	f (41–70)a
	4466.0	4466.5	f (102–138)a
	5613.3	5614.1	f (92–138)b
	14480.5	7239.9 *	f (15–141)b
E	6989.0	3494.0 *	f (41–100)a
	7034.0	3519.3 *	f (76–135)a
	7006.0	3504.7 *	f (76–135)b
	7295.3	3649.4 *	f (102–162)a
	7267.3	3633.7 *	f (102–162)b
F	5597.4	5598.1	f (102–148)a
	5569.4	5569.9	f (102–148)b
	5641.4	5642.1	f (92–138)a
	5613.3	5614.6	f (92–138)b
G	/	/	/
H	11190.9	5595.5 *	f (1–100)b
	10808.4	5403.5 *	f (71–162)a
I	5488.2	5487.5	f (21–69)a
	5430.1	5430.9	f (21–69)b
	11249.0	5624.5 *	f (1–100)a

[a] Monoisotopic mass with single charge, calculated from amino acid sequence. Na and K represent sodium and potassium adduct, respectively.
[b] Observed mass with single charge except for those marked with *, * means double charged mass.
[c] a represents this sequence particularly from β-lactoglobulin A, and b is from β-lactoglobulin B.

behavior between these two models, and their behaviors depend not only on the nature of substrate and enzyme, but also on hydrolytic conditions. For immobilised trypsin, although linear decreases in intact protein dependent on DH were observed at all explored concentrations of NaCl, close to the "one-by-one" model (Fig. 5B2), increasing NaCl concentrations led to a clear decrease in the depletion of intact β-Lg. Specifically, around 18–26% intact protein was hydrolysed in 0 or 0.02 M NaCl at DH ≈ 1%, while it was only 7–10% in 0.1 or 0.5 M NaCl. For free trypsin, a sharp increase in the depletion rate was noted at the initial stage of hydrolysis with 0 or 0.02 M NaCl, during which the increase in DH from 0 to 1% corresponded to a depletion of 30–40% of the intact protein content (Fig. 5A2). This is more in agreement with the "zipper" model, while it seems to be at the "one-by-one" model, when NaCl concentration increased from 0.02 to 0.1 or 0.5 M.

3.3.2. The effects of NaCl on peptide profiles

As illustrated in Fig. 6 and Table 1, peaks located in the range of 45–60 min correspond to intermediate peptides. The accumulation of these peptides was primarily due to the fast breakdown of intact β-Lg and secondarily due to the insufficient subsequent hydrolysis. For both forms of trypsin, 0.1 and 0.5 M NaCl significantly diminished the peaks of intermediate peptides, partly due to the slow hydrolysis of intact β-Lg (Fig. 5A2,B2). These results are in accordance with the findings of Butré, Wierenga, and Gruppen (2012), who reported that in 0.5 M NaCl, the hydrolysate composition of 1–5% WPI (w/w) showed increasing levels in hydrophilic peptides and decreasing amount of hydrophobic peptides, compared with hydrolysis without NaCl.

Regarding peaks eluted before 45 min, most of them were assigned to the final peptides as previously discussed for Fig. 3, thus, these peaks in Fig. 6 are not repeatedly highlighted. The DH-dependent release of these identified peptides is illustrated in Supplementary material Fig. S1 for free trypsin and in Fig. S2 for MITR. Furthermore, Fig. 7 shows the relative amounts of individual peptides based on the relative DH (value R_2). A higher R_2 value of a peptide indicates that this peptide is preferred to be released than other peptides during the hydrolysis. If all final peptides released at the same rate, all R values would be 100%. The effects of NaCl on the release of peptides, as indicated by R_2 values, are summarised in Table 2. For free trypsin, peptides f (61–69/61–70), f (84–91), f (125–125), f (92–101) and f (41–60) were released significantly faster at 0.1 or 0.5 M NaCl. Specifically, at DH 3%, 3-fold higher amount of f (61–69/61–70) and 2-fold higher amounts of f (92–101) and f (41–60) were released with the addition of 0.5 M NaCl, compared with those in 0 and 0.02 M NaCl. Also, f (15–20), f (21–40) and f (125–135) were released much earlier in 0.1 and 0.5 M NaCl. For immobilised trypsin, the addition of 0.1 or 0.5 M NaCl significantly increased f (61–69/61–70) and f (125–135). In addition, levels of f (71–75) and its precursor f (70–75) decreased significantly with the addition of 0.1 and 0.5 M NaCl for immobilised trypsin, while no significant difference in these peptides was observed for free trypsin. In general, the final peptides directly associated with the breakdown of intact protein diminished with increasing NaCl concentrations.

3.3.3. The mechanism underlying the observed influence of NaCl

In contrast to Tris, NaCl is a strong electrolyte and is reported to show quasi-neutral behavior, as both its anion and cation are located in the middle of the Hofmeister series (Salis et al., 2007). Sedlak, Stagg, and Wittung-Stafshede (2008) concluded that at low ion concentrations (<100–200 mM), ions specifically interacted with protein molecules in the manner of Langmuir binding isotherm (specific binding or pairing of ions with proteins), while at ion concentrations > 200 mM, Hofmeister effects dominated (salting out or salting in of proteins). The study exploring ion-pairing effects of NaCl on β-Lg indicates that Na^+ ions pair with carboxylate groups, while Cl^- ions are not significantly enriched near positively charged residues, such as Lys and Arg (Beierlein et al., 2015). At pH 8.7, the overall surface charge of trypsin molecules is theoretically positive, as its PI is 10.1–10.5 (Buck, Vithayathil, Bier, & Nord, 1962). As the electrostatic interactions between free enzyme and substrate molecules dominate (Endo et al., 2016), the pairing effect of Na^+ ions with the carboxylic

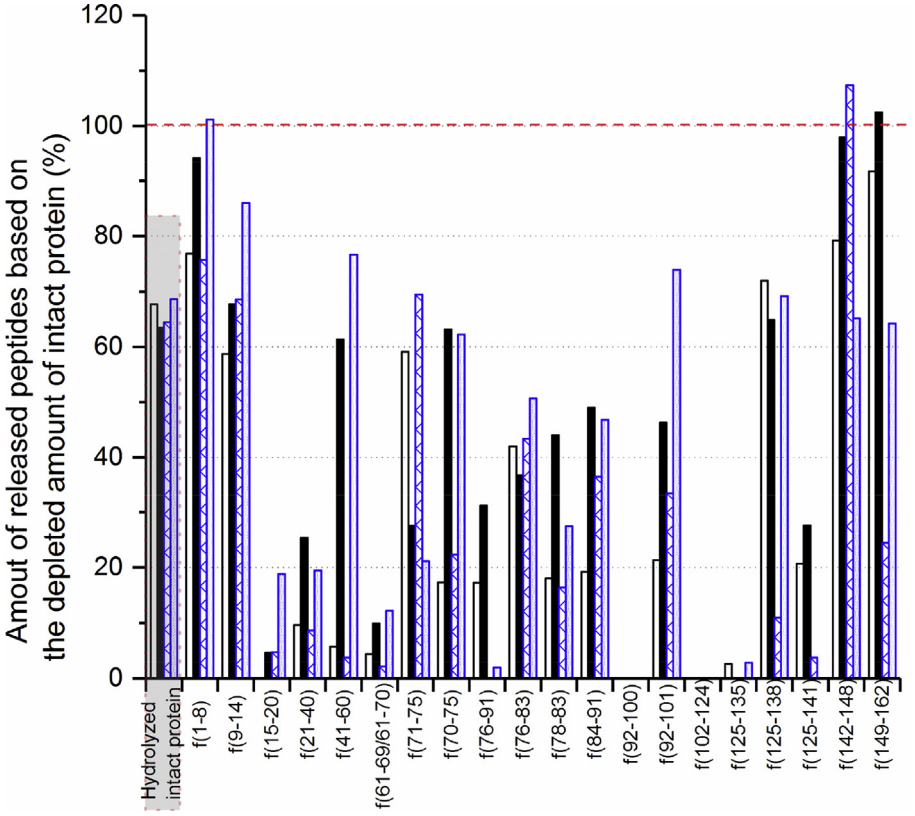

Fig. 4. Effects of Tris buffer on the release of specific peptides. Hollow and black bars represent the cases hydrolysed by free trypsin with and without Tris buffer, respectively. Bars with crossed lines and dots represent the cases hydrolysed by immobilised trypsin with and without Tris buffer, respectively.

Table 2
The effects of salts on the release of peptides.

Effects	Free trypsin	MITR
Effects of Tris[a]		
Increase	f (1–8); f (9–14); f (15–20); f (21–40); f (41–60); f (61–69/61–70); f (78–83); f (84–91); f (92–101)*; f (142–148); f (149–162)	f (1–8); f (9–14); f (15–20); f (21–40); f (41–60); f (61–69/61–70); f (78–83); f (84–91); f (92–101)*; f (125–138)*; f (125–135); f (149–162)
Decrease	f (71–75)	f (71–75); f (142–148)
No significant effect	f (92–100); f (125–138)*; f (125–135)	f (92–100)
Effects of NaCl[b]		
Monotonic decrease	f (1–8); f (9–14)	f (1–8); f (9–14); f (71–75); f (142–148); f (149–162)
Monotonic increase	f (15–20); f (21–40); f (41–60)*; f (61–69/61–70); f (92–101)*; f (125–135); f (125–138)*	f (41–60)*; f (61–69/61–70); f (125–135)
Increase then decrease	f (78–83); f (84–91); f (92–100); f (101–124)*; f (142–148); f (149–162)	f (78–83); f (84–91); f (92–101)*; f (125–138)*
No significant effect	f (71–75)	f (15–20); f (21–40); f (92–100); f (101–124)*

[a] based on the R1 value; [b] based on the R2 value; an asterisk indicates peptides with one missed cleavage site and a negligible amount of the final derivative peptide.

groups of β-Lg at low NaCl concentrations could protect intact protein molecules from hydrolysis by free trypsin. The addition of high concentration of NaCl significantly increased the ionic strength of the reaction media, which might hinder enzyme–substrate interactions through charge shielding. In addition, due to Hofmeister effects, the interactions between molecules of β-Lg increase with increasing concentrations of NaCl, toward dimer formation. This further decreases the depletion of intact protein significantly by both forms of trypsin. Beierlein et al. (2015) provided models on the formation of β-Lg dimmers, including the "lock-and-key", corresponding well to our results. The residues involved in interactions in this model are mainly in termini, and the release of final peptides located in termini decreased significantly at high concentrations of NaCl in this study.

3.4. The mechanism underlying the different influences on free and immobilised trypsin exerted by salts

As previously discussed, Tris and NaCl exerted different influences on free and immobilised trypsin. These differences are probably due to (i) the unconventional flow-through hydrolysis mode and (ii) the surface characteristics of the support used to immobilise trypsin. In this flow-through approach, shear force is a decisive force, driving substrate molecules to immobilised trypsin, since a higher flow rate contributes to a higher hydrolysis efficiency in our previous work (Mao et al., 2017; Mao & Kulozik, 2018). Further, immobilised trypsin attracts substrate molecules at a short distance, depending on the interactions of substrate–enzyme and/or substrate-support surface. In this study, aldehyde-activated

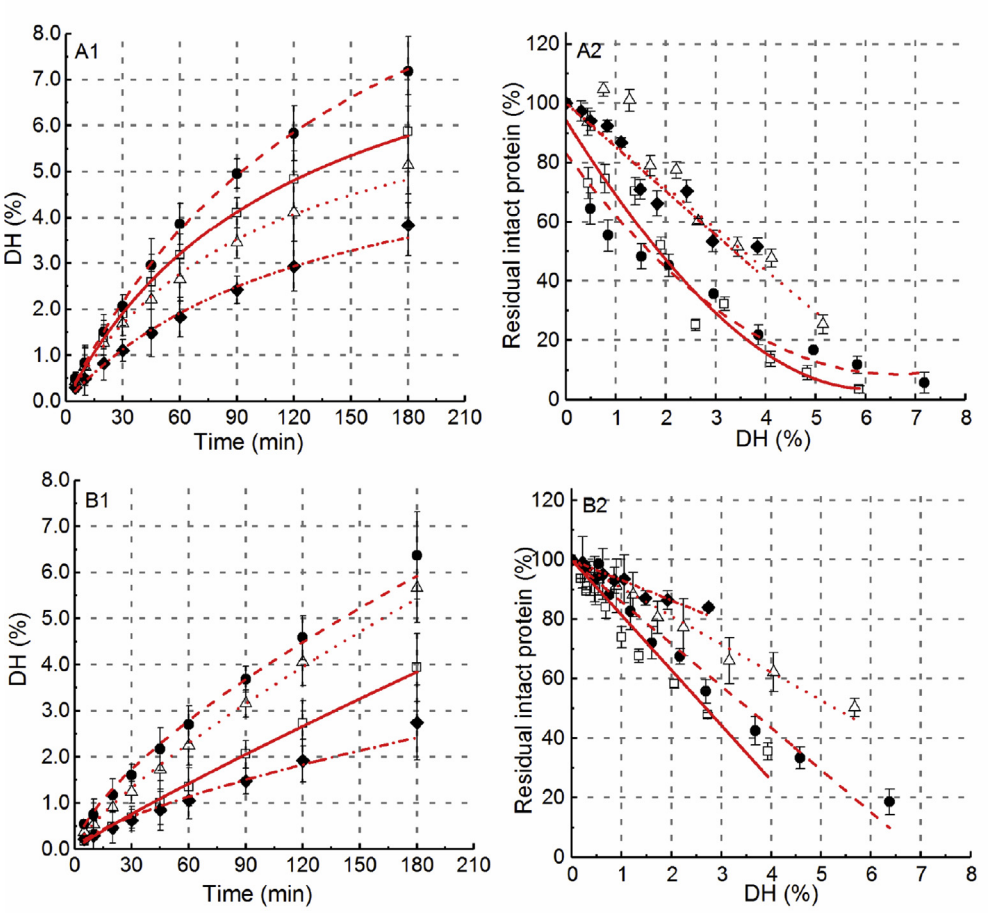

Fig. 5. Effects of 0 M (□), 0.02 M (●), 0.1 M (△), and 0.5 M (◆) NaCl on DH (A1&B1) and the amount of residual native β-lactoglobulin (A2 and B2). A, hydrolysis by free trypsin; B, hydrolysis by MITR. Data values represent mean values of three replicates with a 95% confidence interval.

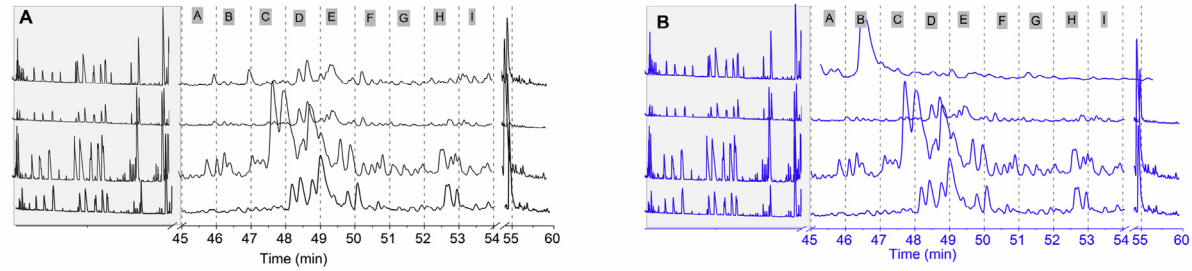

Fig. 6. HPLC profiles of β-lactoglobulin hydrolysates generated from free (A) or immobilised trypsin (B) with increasing NaCl. Samples from top to bottom are hydrolysates produced in 0.5 M, 0.1 M, 0.02 M and 0 M NaCl, respectively.

columns without a spacer linker were used as the immobilisation support, and the surface was preferentially neutral (Naldi, Černigoj, Štrancar, & Bartolini, 2017). In addition, the surface coverage ratio by trypsin molecules was high, about 65 ± 10% (Mao et al., 2017). Thus, the surface charge and hydrophobicity of the support is considered to be same as that of the formed layer of immobilised trypsin molecules. Trypsin was immobilised at pH 5.6 (Mao et al., 2017), where the overall surface charge of trypsin molecules is, in theory, highly positive. Based on the principles of electrostatic interactions, each trypsin molecule probably regulates its position to reach a charge balance with other trypsin molecules during the immobilisation. Hence, the attractive force between substrate molecules and immobilised trypsin is probably different from that for free trypsin, which is mainly dependent on electrostatic interactions (Evnin et al., 1990). Our results corroborate this prediction. For instance, negatively-charged precursor peptides f (125–141), f (125–138) and f (102–138) were much less attracted to immobilised trypsin than for free trypsin. Alternatively, the hydrophobic precursor peptides were generally preferred over hydrophilic peptides for immobilised trypsin. Therefore, hydrophobic interactions are implicated as the attracting forces at a short distance, determining the interactions between substrate molecules and immobilised trypsin. With the addition of salts, ions may provide charge shielding/electrical double layer or stoichiometric

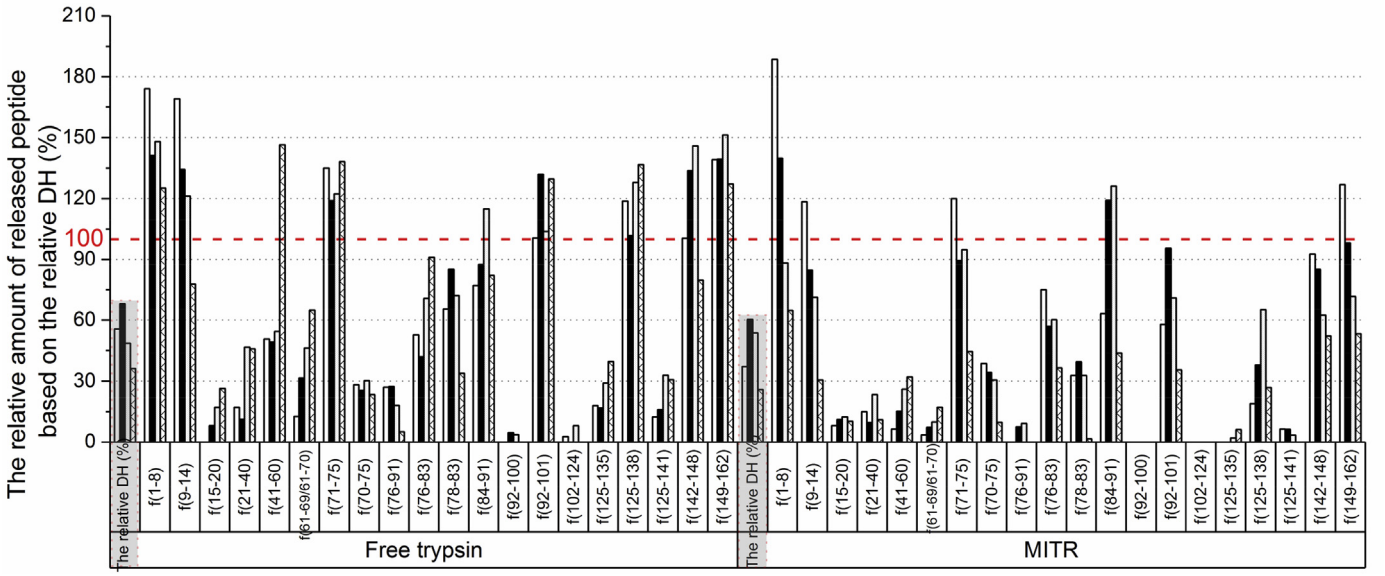

Fig. 7. Effects of NaCl on the release of specific peptides. Hollow bars, black bars, bars with dots, and bars with crossed lines represent the hydrolysis of β-lactoglobulin in 0, 0.02, 0.1 and 0.5 M NaCl, respectively.

ion binding on the support surface in MITR, which is used for column chromatography to suppress nonspecific adsorption of proteins to the column surface (Tsumoto et al., 2007). Thus, due to the ions binding on the surface of immobilisation support, this support may exert an additional repulsive force on intact protein and/or an attractive force on certain peptides.

4. Conclusions

In this study, the influence of salts was explored comparatively between free and immobilised trypsin for the hydrolysis of β-Lg. Compared with the hydrolysis without additional NaCl, DH increased faster for both forms of trypsin with the presence of 0.02 NaCl, especially for immobilised trypsin, DH increased in 0.1 M NaCl as well. Regarding the hydrolysis of intact protein, increasing NaCl concentrations significantly reduced the depletion rate for both forms of trypsin, while 0.1 M Tris had no significant influence on hydrolysing intact protein. In addition, both forms of trypsin preferentially hydrolysed certain intermediate peptides in the presence of salts, depending on the type and concentration of salts as well as on the form of trypsin. This study followed the change in peptide profiles at the early stage of hydrolysis, and provided the possibility of a partial hydrolysis of β-Lg with desired peptide compositions, for example, a hydrolysate with a maximum accumulation of certain functional intermediate peptides.

Generally, the hydrolysis efficiency and hydrolysate profiles can be regulated by adding different salts at varied concentrations, since salts significantly influence the interactions between proteins/peptides and enzymes. This study may provide some hints on the choices of buffers or salts to regulate the hydrolysis process, improve the hydrolysis efficiency or obtain desired product profiles. In future studies, the investigation of a hydrolysis medium with more complex salt compositions, such as milk salts, could be quite interesting. Milk salts contain calcium, magnesium, sodium, and potassium as the main cations and inorganic phosphate, citrate, and chloride as the main anions. The hydrolysis of milk proteins without the removal or with the addition of these salts could provide consumers with extra benefits, as these minerals are essential for bone growth and development. However, it is necessary to clarify whether these minerals will considerably influence the hydrolysis efficiency and/or the resulting hydrolysate profiles.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.02.006.

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