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ORIGINAL PAPER

Application of AprX from *Pseudomonas paralactis* for the improvement of the emulsifying properties of milk, plant and insect protein and estimation of their hydrolysate's bitter potential

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Abstract

Protein properties can be modified by selective enzymatic hydrolysis. In this study, the alkaline metalloendopeptidase AprX (Serralysin; EC 3.4.24.40) from *Pseudomonas paralactis* was used for the tailored hydrolysis of different food proteins resulting in the production of protein hydrolysates with improved emulsifying properties. Sodium caseinate, wheat gluten and buffalo worm protein were used for AprX hydrolysis at 40 °C and pH 8 to cover a spectrum of different protein sources. A maximum degree of hydrolysis (DH) of 13.1 ± 0.2%, 14.2 ± 0.1% and 20.7 ± 0.1% was reached for sodium caseinate, wheat gluten and the worm protein, respectively. The corresponding hydrolysate properties were analyzed regarding their particle size, peptide composition, solubility, viscosity, surface hydrophobicity and interfacial tension. The emulsifying properties were investigated by the oil-droplet size, ζ-potential and stability of emulsions prepared from the hydrolysates. Using partially hydrolyzed sodium caseinate (DH = 10.6%) as an emulsifier lead to an eightfold increase of the emulsion stability ($t_{1/2}$ = 180 ± 0 min) compared to unhydrolyzed sodium caseinate. The emulsion stability using wheat gluten hydrolysates (DH = 11.9%) was increased 30-fold ($t_{1/2}$ = 45 ± 5 min). Simultaneously, the solubility of gluten was increased by 60%. Buffalo worm hydrolysates (DH = 14.6%) had a twofold ($t_{1/2}$ = 85 ± 5 min) increased emulsion stability. In conclusion, AprX can be used to improve the solubility and emulsifying properties of food proteins at a relatively high DH.

Keywords Sodium caseinate · Wheat gluten · Buffalo worm protein · Peptide · Technofunctionality · Enzymatic hydrolysis

Introduction

In terms of future nutrition, dietary proteins that are readily available in high quantities and providing essential amino acids are desirable [1]. In addition, food proteins are characterized by their technofunctionality, which plays an important role during food production and in the final product. In particular, technofunctionality comprises, for example, the water-holding capacity, oil- and fat-binding properties, gelling properties and the ability to stabilize oil–water or gas–water interfaces [2].

Proteins are also classified as surface active agents or short (bio-)surfactants due to their ability to interact with and stabilize interfaces [3]. As an example, sodium caseinate derived from milk is already used as an effective surfactant and can be applied in ice cream or other frozen desserts, and in high protein or protein enhancement products [4, 5]. Sodium caseinate consists of four protein fractions: α_{S1}-, α_{S2}-, β- and κ-casein [6]. The emulsifying properties of sodium caseinate are mainly caused by the high adsorption rate of β-casein to the interface and by the molecular flexibility of the caseins (no tertiary structure) that promote the adsorption of the proteins at the interfaces [2, 7].

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While proteins derived from animal origin, such as caseinates, are still the main protein source in most diets, proteins from plant material and insects represent attractive alternatives. Alternative protein sources are gaining more attention due to the increasing world population, and can play a major role in terms of nutrient delivery [1, 8]. Wheat gluten, for instance, is a byproduct of wheat starch production and represents a cheap alternative to animal protein [9]. It is formed of 45–50% (w/w) polymeric glutenins and 50–55% (w/w) monomeric gliadins [10]. Unhydrolyzed gluten shows poor emulsifying properties due to its poor water solubility [9, 11]. With an increasing number of recent regulatory approvals, edible insect proteins can be considered as one alternative protein source to secure future protein needs, revealing promising nutritional quality as they include most essential amino acids and a high protein digestibility [12–19]. One example of edible insects is the buffalo worm or lesser mealworm (*Alphitobius diaperinus*) with a protein content of approximately 58% [20]. The nutritional quality of proteins derived from a buffalo worm is found within the same range as soy protein or caseins [14]. As an additional benefit, a variety of biofunctional compounds has been described for insects, such as chitin, polyphenols, antioxidative enzymes and bioactive peptides [15]. This indicates a versatile use of insect ingredients in food products in the future [14]. However, insect protein is currently regarded with low acceptance in Western markets and, thus, will need more positive exposure to the consumers, for example, as a functional ingredient with low inclusion rates [21–23]. Regarding the technofunctional properties of insect proteins, they showed good gelation behavior but poor foamability, with a half-life of less than 6 min [20]. The ability to stabilize oil/water interfaces in correlation with the protein concentration applied has been shown previously [24], indicating the possible use of insect proteins as an emulsifier in food products. Application possibilities for insect proteins might include baking applications [5], the production of meat analogues [25], high protein products and protein enhancement products [5].

Enzymatic hydrolysis with peptidases is an established tool in the food industry to alter the technofunctional properties of food proteins [26, 27]. By such hydrolysis, the peptide bonds of the proteins are cleaved and peptides with a decreased molecular weight as well as free amino acids are produced [3]. This generally causes the exposure of hydrophobic groups from the inner protein structure, an increased number of ionizable groups, an increased solubility, a decreased viscosity and, therefore, a modified behavior at interfaces (emulsions, foam) [28, 29]. Compared to traditional emulsifiers used for interfacial stability, technofunctional hydrolysates may increase the nutritional value of foods (such as increased protein content) and have the potential for a 'cleaner-labeling' (labeling as 'protein hydrolysates') [30].

Besides the protein source, the technofunctionality achieved by the enzymatic hydrolysis depends on the selectivity of the enzyme used. Our workgroup described a heat-stable endopeptidase from *Pseudomonas paralactis* in a previous work [31]. The enzyme belongs to the class of metalloendopeptidases and was classified as AprX (Serralysin; EC 3.4.24.40). As such, the substrate selectivity of AprX is described as broad, with a slight preference of basic (K, R, H) or aromatic (Y, F, W) residues at the P1 position and nonpolar aliphatic amino acid residues (V, A, I, L, M, F, Q) at the P1' position [32, 33]. Cleavage sites in casein are mainly described in the hydrophobic areas of the protein [32]. Thus, a large variety of peptides are assumed to be released from the food proteins, which might have positive properties in terms of solubility and emulsification. In contrast to most other peptidases, AprX was described as heat stable, with the ability to withstand commonly used ultra-high temperature, but lower temperature treatment at around 55 °C inactivates the enzyme [31]. A low inactivation temperature is preferable for protein treatment in the food industry, as it reduces the risk of high-temperature impact, which could otherwise alter both the hydrolysis success and the resulting hydrolysates.

Therefore, the aim of this study was to investigate the potential of AprX as a tool to modify the technofunctionality of different food proteins. Due to its selectivity and temperature characteristics, AprX was applied to modify the emulsifying properties of three protein sources (sodium caseinate, gluten and buffalo worm protein). The resulting hydrolysates were investigated in a multilevel approach, considering the bulk properties of the hydrolysate from a molecular scale to the appearance of end products.

Materials & methods

The chemicals used in this work were of analytical grade and purchased from Sigma Aldrich (Schnelldorf, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany) or AppliChem (Darmstadt, Germany). Sodium caseinate powder was obtained from FrieslandCampina (Amersfoort, Netherlands). Its specifications were: 90.6% (w/w) protein, < 1% lactose (w/w), < 1% fat (w/w) and 4.1% (w/w) minerals. Wheat gluten was obtained from Nestlé PTC (Singen, Germany) and was composed of 83% (w/w) protein, < 10% (w/w) carbohydrates, < 7% (w/w) fat and < 8% (w/w) water. The buffalo worm (*Alphitobius diaperinus*) protein flour was purchased from Entopure–Proti-farm® (Ermelo, Netherlands) with the specifications of 58.6% (w/w) protein, 6.7% (w/w) carbohydrates, 24.7% (w/w) fat and 5.2% (w/w) fibers. A column (XK 16/40, 1.6 × 40 cm) from GE (Munich, Germany) was filled with Biofox 40 S resin (cation exchange chromatography material) from Knauer Wissenschaftliche Geräte GmbH (Berlin, Germany) for AprX purification. The MINI-PROTEAN system (Bio-rad Laboratories GmbH, München, Germany) was used for polyacrylamide gel electrophoresis. Molecular weight markers were purchased from New England Biolabs (NEB; Frankfurt, Germany). The medium chain triacylglyceride mixture Mygliol 812N® was obtained from Sasol Germany GmbH (Brunsbüttel, Germany).

Production of AprX from *Pseudomonas paralactis*

Pseudomonas paralactis DSM 29164 was cultivated for 45 h in 100% (v/v) skimmed UHT milk (0.3% fat, gut & günstig, EDEKA, Hamburg, Germany) at 20 °C using a parallel bioreactor system, as described by Volk *et al*. [34, 35]. The supernatant containing the endopeptidase AprX was purified by cation exchange chromatography using an ÄKTA-FPLC system (GE, München, Germany), according to Glück *et al*. [31]. The purity of the endopeptidase was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, according to the method of Laemmli [36]. Accordingly, a 12.5% (w/v) cross-linked separating polyacrylamide gel and a 6% (w/v) stacking gel were used. After separation, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the protein bands and the molecular mass marker (2–212 kDa; New England Biolabs, Ipswich, MA, USA) [37] (SI, Fig. S1). The protein content was determined according to the method of Bradford [38], using bovine serum albumin as a protein standard. The highly purified AprX had a specific endopeptidase activity of 528 nkat/mg (determination using *ortho*-phtaldialdehyde method and 2.5% (w/v) sodium caseinate as a substrate at 40 °C and pH 6.7) with a yield of 58% and purification factor of 3.7 after cation exchange chromatography.

Determination of the endopeptidase activity

The endopeptidase activity of AprX was determined at the same conditions used for the later hydrolysis (40 °C, pH 8.0) and with all three food proteins as a standardized substrate solution (90 $g_{Protein}$ L^{-1}). The determination of newly formed *α*-amino groups was performed by using the *ortho*-phthaldialdehyd method, as described by Nielsen *et al*. [39, 40] and Baur *et al*. [36]. A solution of L-serine (0.08–5.0 mM) was used for calibration. One katal of peptidase activity was defined as the amount of peptidase that is required to release 1 mol serine-equivalent per second.

Hydrolysis process

Hydrolysis of the food proteins was conducted in separate batch processes. The food proteins (standardized to 90 $g_{Protein}$ L^{-1}, hydrolysis volume 0.75 L of sodium caseinate and wheat gluten, 1.03 L of buffalo worm protein) were dispersed in H_2O_d and the pH was adjusted to 8.0 using 2 M NaOH. Before hydrolysis, the colloidally dissolved buffalo worm protein was defatted by centrifugation (three times, 20 min, 10,000 rpm, 4 °C). The resulting fat layer was removed. Sodium azide [0.1% (w/v)] was added to the solutions to avoid microbial growth during the process. The hydrolysis was started by the addition of the initial endopeptidase activity, either 2 nkat mL^{-1} [wheat gluten (total activity: 1.5 mkat) and buffalo worm protein (total activity: 2.06 mkat)] or 19 nkat mL^{-1} [sodium caseinate (total activity: 14.25 mkat)]. The endopeptidase activity added was determined in preliminary experiments to reach the maximum degree of hydrolysis (DH) in a defined time frame (36 h). The pH of the protein solutions was measured (pH 8.0) and adjusted automatically using 2 M NaOH. Samples (80 mL) were taken over 48 h and subsequently heated at 55 °C for 30 min to inactivate the endopeptidase. Heat treatment conditions were identified after measuring residual AprX activity in preliminary experiments. The samples were stored at– 80 °C until further analysis. Additionally, samples (2.5 mL) were taken directly after the endopeptidase addition, after 24 h and at the end of hydrolysis (36 h). These samples were not heated and were used for the determination of the residual endopeptidase activity using azocasein (5 g L^{-1} in MOPS, 50 mM, pH 8.0) as a substrate as described by Charney and Tomarelli [40, 41] and Baur *et al*. [40, 41].

Analysis of the hydrolysis product

The heated samples from hydrolysis were analyzed regarding their DH as well as their macro- and micromolecular properties in oil-in-water systems. The samples were diluted to a protein content of 9 $g_{Protein}L^{-1}$ using H_2O_d, unless stated otherwise.

Degree of hydrolysis

Firstly, sodium dodecyl sulfate (SDS) (10% (w/v), 50 µL) was added to the samples (450 µL) to solubilize the proteins. The number of free α-amino acid groups was then determined by a derivatization with *ortho*-phthaldialdehyd, as described previously (see Section "Determination of the endopeptidase activity"), and calculated, as described by Ewert *et al*. [42, 43].

Peptide pattern

A 16% tris-tricine gel containing 6 M urea, according to Schägger [44], was used to separate the peptides formed during hydrolysis. Proteins/peptides (40 µg per lane) were stained using Coomassie Brilliant Blue R-250 [37]. The Spectra™ multicolor, low range protein ladder (Thermo-Fisher Scientific, Waltham, MA, USA) was used for the molecular size determination. An additional SDS-PAGE to visualize the full profile, including larger peptides, was conducted in the case of buffalo worm protein hydrolysis, as described previously (see Section "Production of AprX from Pseudomonas paralactis").

Particle size of the hydrolysates

The average particle size of the hydrolysates was determined using a NanoZetasizer (Malvern Instruments, USA), according to Ewert *et al*. [45], at a refractive index of 1.445. The samples were diluted with H_2O_d (protein content: 1.8 g L^{-1}) and the pH was adjusted (7.0) using HCl (0.1 M).

Peptide composition of the hydrolysates

The peptides found in the hydrolysates were investigated by mass spectrometric analysis by the Core Facility for Mass Spectrometry (University of Hohenheim), as described previously [45]. The peptides found in the hydrolysates were aligned using Mascot 2.3 (Matrix Science, London, United Kingdom) for searching the database UniProt [45]. In the case of the Buffalo worm protein hydrolysates, the proteosome of *Drosophila melanogaster* was used for the evaluation to achieve a satisfying sequence coverage of the peptide mixture. Only single proteins were described for *Alphitobius diaperinus* and, therefore, peptides generated in the hydrolysis might have been missed during the evaluation.

The grand average of hydropathy (GRAVY) was determined for each detected peptide formed during hydrolysis. The GRAVY is expressed as the sum of the hydropathy value of the amino acids present in a peptide divided by the number of amino acid residues in the sequence [45, 46]. The potential bitterness of the resulting peptides was determined according to Ney [47]. Consequently, the Q-value of each peptide was calculated. Additionally, peptides derived from sodium caseinate were compared to those known to provide a bitter potential in dairy products [48, 49].

Apparent viscosity and interfacial tension of the hydrolysates

The apparent dynamic viscosity was investigated using a programmable Brookfield DV-II + ProViscometer (Brookfield Engineering Laboratories, Middleboro, MA, USA) equipped with a small sample adapter and a SC4-18 spindle, as described previously [45]. Samples taken during the hydrolysis of sodium caseinate and wheat gluten (7 mL) were measured undiluted. Samples taken during the hydrolysis of buffalo worm protein were diluted with H_2O_d to a standardized protein content of 0.9% (w/v) due to the shear-related viscosity increase during the measurement. Additionally, a shear/time-dependent (30 min) analysis was done for wheat gluten and its hydrolysates at 20 °C and 200 rpm.

The interfacial tension of the hydrolysates was determined using a drop-shaped analyzer (DSA G-10, MK2, Krüss, USA) at the oil–water interface. Here, a pendant drop of the sample solution was formed at the tip of a syringe. The drop was surrounded by the oil phase. Based on the shape of the drop, the interfacial tension was calculated according to the Young–Laplace equation [50]. The hydrolysates were filled into a syringe (tip diameter 1.26 mm). The tip of the syringe was submerged into a cuvette filled with Mygliol 812 N® as the oil phase. The syringe and cuvette were positioned between a light source and a high-speed charge-coupled device camera [50]. The interfacial tension was measured over a period of 40 min. The protein concentration of the hydrolysates was 0.045% (w/v). Tap water and the emulsifier Tween 20 (concentration 0.045% (w/v)) were investigated as references. The respective densities for the calculation of the interfacial tension were determined using a digital density meter (DMA 35N, Anton Paar Physica, Ostfildern-Scharnhausen, Germany).

Solubility of the hydrolysates

The solubility of the hydrolysates was determined gravimetrically [43]. Therefore, samples (2 mL) were centrifuged (30 min, 13,000 × *g*, 4 °C). The resulting supernatant (1 mL) was transferred to a weighted reaction tube. Samples were then dried by vacuum centrifugation (1 mbar, 60 °C, 4–7 h). Afterwards, samples were weighted, and the solubility (protein concentration in supernatant) was calculated using the following equation:

$$Solubility\,[\%] = \frac{m_{drymass} - m_{emptyreactiontube}}{m_{substrate}} \times 100$$

where: $m_{dry\ mass}$ [mg]: mass of the dried supernatant after vacuum centrifugation, $m_{empty\ reaction\ tube}$ [mg]: mass of the empty reaction tube, $m_{substrate}$ [mg]: mass dissolved for the standardized food protein solution of 90 $g_{Protein}$ L^{-1}

Relative surface hydrophobicity of the hydrolysates

The relative surface hydrophobicity S_0 was determined using PRODAN (N,N-dimethyl-6-propionyl-2-naphtalene), according to Ewert *et al*. [45]. Hydrolysates from sodium caseinate were diluted to a protein concentration of 0.03 to 1.8% (w/v). Wheat gluten hydrolysates were diluted in a range of 0.04 to 9.0% (w/v), whereas the hydrolysates of buffalo worm protein were diluted from 0.002 to 0.1% (w/v). The relative fluorescence intensity of the samples was calculated as described by Alizadeh-Pasdar and Li-Chan [51] and fitted over the protein concentration. The slope of this plot represents the surface hydrophobicity. The R^2 of each plot was ≥ 0.95.

Emulsion preparation and analysis

Emulsions of the hydrolysates were prepared, as described by Ewert *et al*. [45]. Briefly, oil-in-water emulsions were prepared using a high shear blender (SilentCrusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) with a 12 F/M nozzle. The hydrolysis samples acted as the continuous phase. The pH was adjusted to 7.0 using HCl (0.5 M). The solutions (80 g) were blended with Mygliol 812 N® (20 g), which acted as a disperse phase, at 20,000 rpm for 6 min. The emulsions were analyzed immediately regarding the oil-droplet size and electrokinetic potential (ζ-potential), as described previously [45, 52]. Slight modifications took place for the measurement of the electrokinetic potential. Here, the samples from the sodium caseinate and buffalo worm protein were diluted to a protein concentration of 0.045% (w/v), whereas samples from the wheat gluten hydrolysis were diluted to 0.036% (w/v) using H_2O_d. The direction and velocity of the droplet movement in the electric field applied was detected and the ζ- potential was calculated automatically using the Smoluchowski equation [27, 53].

Colored emulsions were prepared, as described by Ewert *et al*. [45], to determine the emulsion stability. Accordingly, methylene blue (20 mg L^{-1}) was used to stain the continuous phase, while the disperse phase was stained using bell pepper extract (0.5% (v/v); Paprika-Extrakt 30 TFE, Gewürzmüller, Salzburg, Austria), resulting in a red to orange color. The emulsions were blended using a high shear blender at 20,000 rpm for 6 min turning them into a green color [45]. The phase separation of the emulsions was analyzed semi-automatically in volumetric flasks (50 mL) and the half-life was calculated, as described previously [45].

Statistical analysis

Standard deviation was used for the data evaluation and calculated with Excel (Microsoft, Redmond, USA). All experiments were conducted at least in duplicate with three independent measurements, unless stated otherwise.

Results

The endopeptidase AprX from *Pseudomonas paralactis* was used for the hydrolysis of three food protein substrates in this study. Sodium caseinate, wheat gluten and buffalo worm protein were used as substrates representing proteins from animal, plant and insect sources, respectively, to cover different protein sources. The hydrolysates produced were investigated in detail regarding their peptide characteristics and possible application as surfactants.

Hydrolysis process

The highly purified endopeptidase AprX from *P. paralactis* was used for the hydrolysis of sodium caseinate, wheat gluten and buffalo worm protein (SI, Fig. S1). The endopeptidase activity had to be adapted in preliminary experiments to reach the maximum DH in a defined time frame (36 h) (data not shown). Residual AprX activity has not been detected after heat treatment at 55 °C for 30 min in preliminary experiments (data not shown). The kinetics of the three hydrolyses are shown in Fig. 1. The DH increased directly after the addition of AprX to 4.2 ± 0.01% (sodium caseinate), 3.4 ± 0.02% (wheat gluten) and 2.3 ± 0.05% (buffalo worm protein) (Fig. 1). The maximal DH was reached after 36 h at 13.1 ± 0.2% (sodium caseinate), at 14.2 ± 0.1% (gluten) and at 20.7 ± 0.1% (buffalo worm protein).

Protein hydrolysis yielded a substantial DH of up to 20%, with buffalo worm protein exhibiting the highest DH compared to sodium caseinate and wheat gluten hydrolyses. This suggests a higher abundance of accessible cleavage sites in buffalo worm protein, possibly attributable to the presence of hydrophobic amino acid residues in sodium caseinate and wheat gluten. The residual AprX activity at the end of hydrolysis was 57.2 ± 5.6% (sodium caseinate), 19.7 ± 5.7% (wheat gluten) and 13.9 ± 3.7% (buffalo worm protein), indicating that a potential lack of residual activity did not limit the hydrolysis process.

The partial hydrolysis of the protein sources was confirmed by Tris-Tricine SDS-PAGE for a small molecular weight range revealing the degradation of large peptides into smaller peptides (< 10 kDa) (SI, Fig. S2). All casein fractions (molecular weight (MW) between 19 and 24 kDa) were hydrolyzed during the process, which resulted in

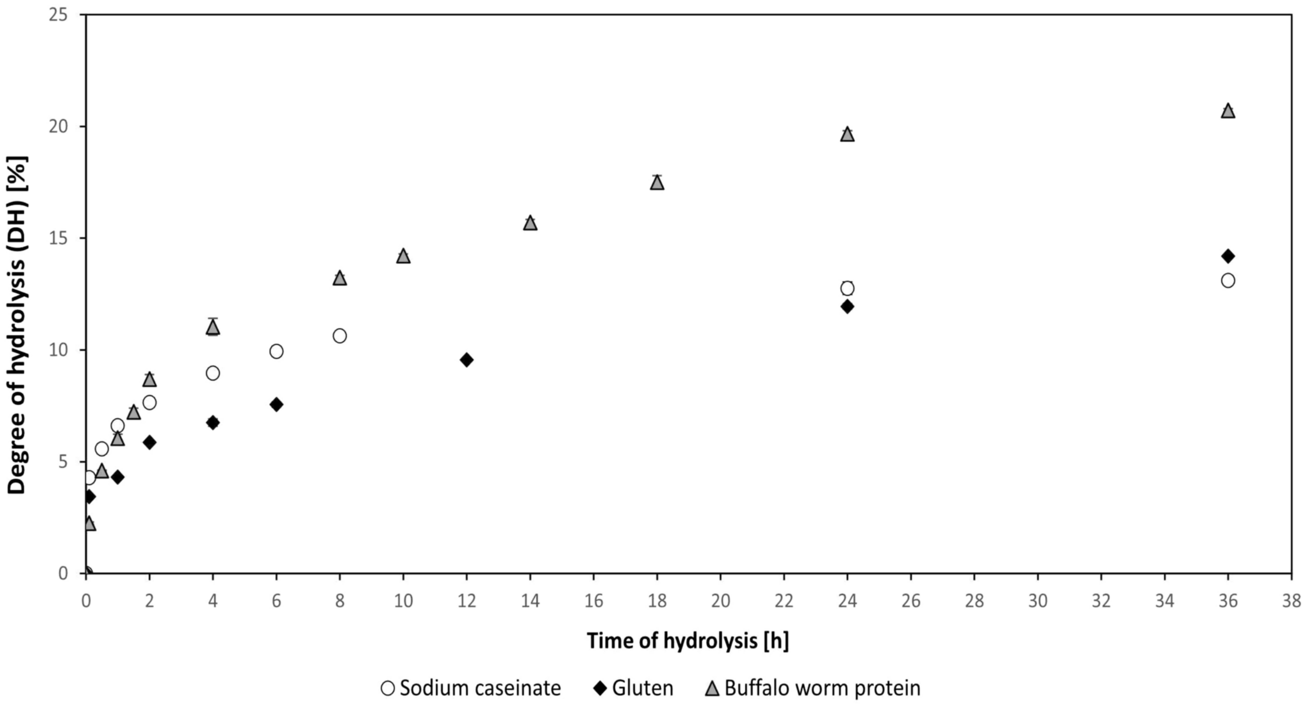

Fig. 1 Hydrolysis progress of sodium caseinate, wheat gluten and buffalo worm protein with AprX (substrate concentration: 90 $g_{Protein}$ L^{-1} in H_2O_d, T = 40 °C, pH 8.0, adapted initial specific AprX activity: sodium caseinate 0.2 mkat g^{-1}, wheat gluten / buffalo worm protein 0.02 mkat g^{-1}, DH determined via *ortho*-phthaldialdehyde assay)

smaller peptides between 5 and 15 kDa until a DH of 10.6% and peptides < 2 kDa at a DH of 12.8%. Wheat gluten was colloidally dissolved during the hydrolyses due to its poor solubility in water phases. Consequently, the intensity of peptides visible on the tricine SDS gel increased due to the solubilization of undissolved protein by the hydrolysis (SI, Fig. S2). Wheat gluten consists of polymeric glutenin (MW 60–140 kDa) and monomeric gliadin (MW $\alpha/\beta/\gamma$-gliadins 30–45 kDa) that were hydrolyzed during the process, resulting in a higher intensity of smaller peptides (< 15 kDa). The buffalo worm protein used represented a heterogenous mixture of peptides/proteins of different molecular sizes, ranging from 10–250 kDa. Due to the heterogenous mixture of the protein fragments, no preference of AprX towards a particular protein fragment was observed (SI, Fig. S2).

The peptide composition of three different DH (8.7–9.6%; 10.6–14.2%; maximal DH) in the three hydrolyses experiments were analyzed further using mass spectrometry (see SI, Tab. S3). All peptides present in the three protein samples at time zero were removed from the analytical evaluation. High sequence coverage was obtained for the samples of sodium caseinate (α_{S1}-casein: 64–75%, α_{S2}-casein: 41–60%, β-casein: 77–92%, κ-casein: 46–72%) and wheat gluten (α/β-gliadin: 26–46%, γ-gliadin: 18–39%, glutenin: 2.6–61%). Although AprX cleaved all protein fractions in sodium caseinate and wheat gluten, it can be assumed that AprX preferentially cleaved β-casein and glutenin. The sequence coverage in case of wheat gluten was low at the beginning of the hydrolysis due to its low solubility in water but increased during the hydrolysis process. In the case of buffalo worm protein, the peptides were aligned with the proteosome of *Drosophila melanogaster* (coverage: 7.7–35%), due to the lack of the characterization of the proteosome of *Alphitobius diaperinus*, and *D. melanogaster* being the insect proteosome best characterized. Due to the increasing sequence coverage in buffalo worm protein hydrolysates, it can be assumed that the solubility was increased over the course of hydrolysis.

Peptides found in sodium caseinate samples were sized mainly between 0.74 and 4.4 kDa. A total of 100 peptides were found for α_{S1}-casein, 62 for α_{S2}-casein, 118 for β-casein and 33 for κ-casein at the end of the hydrolysis. The highest number of peptides derived from α_{S1}- casein and β-casein. The maximum number of peptides was found at a DH of 8.97% for α_{S2}-casein (69), β-casein (148) and κ-casein (57). The maximum number of peptides released from α_{S1}-casein (176) was detected at a DH of 10.6%. The molecular size of the peptides found in wheat gluten hydrolysates was between 0.8 and 2.6 kDa. At the end of hydrolysis, 55 peptides were found for α/β-gliadin, 42 for γ-gliadin and 101 for glutenin. The maximum number of peptides was found for all fractions at a DH of 9.6% (α/β-gliadin 99, γ-gliadin 97 and glutenin 212). The molecular size of peptides derived from buffalo worm protein was between

0.79 and 2.9 kDa. At the end of hydrolysis, 338 unique peptides had been detected, while the maximum number of peptides was found at a DH of 14.2% (392 peptides). A large variability of peptides was found in all samples. The maximum number of peptides in all hydrolysates in the different sources was detected in a DH range of 8.97–14.2% and decreasing until the end of hydrolysis. This could be due to the removal of smaller peptides present at the end of the hydrolysis (36 h) during sample preparation or too small size for the detection.

Hydrophobicity

The net hydrophobicity of the hydrolysates and the hydrophobicity of the individual peptides were analyzed. The net hydrophobicity (surface hydrophobicity S_0) is influenced mainly by the folding of the proteins and exposure of hydrophilic/hydrophobic groups to the protein surface. The hydrophobicity of single peptides is influenced by the hydrophobicity indices of the single amino acids and gives an overall value for each peptide found via mass spectrometry.

Regarding the net hydrophobicity of the hydrolysates, the relative surface hydrophobicity S_0 was altered for the proteins from all three sources after treatment with AprX from *P. paralactis* (Table 1). The hydrophobicity of sodium caseinate decreased linearly from 740 ± 45 (equals 100%) to 255 ± 11 (– 66%) at the maximum DH (13.1%) (SI, Fig. S3). This could be caused by the release of smaller peptides with a higher amount of hydrophilic amino acid residues exposed at the protein surface. Simultaneously, the hydrophobicity of the peptides found in sodium caseinate samples was decreased, indicated by a lower GRAVY compared to the initial protein (range 39–40%, initial GRAVY α_{S1}-casein:– 0.48, α_{S2}-casein:– 0.70, β-casein:– 0.15, κ-casein:– 0.29) (SI, Tab. S1).

Hydrolysates of buffalo worm protein decreased in their surface hydrophobicity from 7460 ± 49 (equals 100%) to 5313 ± 110 (– 29%; DH = 20.7%) (SI, Fig. S3), which is similar to sodium caseinate. More than 50% of all peptides found showed a GRAVY greater than the initial protein (GRAVY– 0.86) and, therefore, an increased hydrophobicity due to the release of peptides which are likely to interact at the interface, highlighting the amphiphilic character of the proteins, with hydrophobic patches of the protein probably located inside the peptide structures.

The surface hydrophobicity of hydrolysates released from wheat gluten increased from 155 ± 9 (equals 100%) to 1910 ± 33 (+ 94%; DH = 15.3%) (SI, Fig. S3). This also shows the increased solubility, as observed from the tricine SDS-PAGE results. While the hydrophobic amino acid residues might not have been accessible for the dye in the

Table 1 Technofunctional properties of untreated sodium caseinate, wheat gluten and buffalo worm protein solutions and at different degrees of hydrolysis using AprX from *P. paralactis*

Parameter	Sodium caseinate				Wheat gluten				Buffalo worm protein			
DH [%]	0	8.97	10.6	13.1	0	9.6	11.9	15.3	0	8.7	14.2	20.7
Average particle size [µm]	0.25 ± 0.03	0.20 ± 0.02	0.24 ± 0.06	0.56 ± 0.24	1.34 ± 0.23	1.55 ± 0.17	1.55 ± 0.09	1.80 ± 0.18	3.83 ± 0.56	2.46 ± 0.24	1.95 ± 0.08	2.08 ± 0.26
Protein solubility [%]	86.5 ± 1.8	90.6 ± 0.9	91.3 ± 0.2	90.9 ± 0.2	11.9 ± 0.1	71.1 ± 0.8	71.9 ± 0.9	74.3 ± 0.3	28.1 ± 0.1	33.2 ± 0.6	35.9 ± 0.2	38.6 ± 0.3
Viscosity η [mPa*s]	43.1 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	11.6 ± 0.6	3.4 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
Relative hydrophobicity index S_0 [–]	740 ± 45	374 ± 21	326 ± 20	255 ± 11	155 ± 9	1605 ± 46	1924 ± 195	1908 ± 33	7460 ± 49	5781 ± 254	5458 ± 158	5313 ± 109

insoluble protein, they are more exposed to the peptide surface of the hydrolysates, leading to the increase in hydrophobicity. An increase in hydrophobic peptides by 21–31% was found during hydrolysis via mass spectrometric analysis. However, most peptides (~70–80%, SI, Tab. S1) were more hydrophilic than the initial protein, which was probably related to the removal of large insoluble hydrophobic peptides during the sample preparation.

Solubility, particle size and viscosity of the peptides

The initial solubility of sodium caseinate was 86.5 ± 1.8% and increased during the hydrolysis by AprX up to 90.9 ± 0.2% due to the generation of smaller peptides (Table 1, SI, Fig. S4). The solubility of the wheat gluten hydrolysates increased from 11.9 ± 0.1% to 74.3 ± 0.3% until the end of hydrolysis (36 h) (SI, Fig. S4). The solubility of buffalo worm protein increased from an initial 28.1 ± 0.1% to 38.6 ± 0.3% after hydrolysis (SI, Fig. S4). The increase in solubility was caused by the generation of smaller, more hydrophilic peptides in all cases.

Furthermore, the particle size of the proteins and peptides in the hydrolysates was investigated. After the maximum particle size of wheat gluten hydrolysates was reached at 2.33 ± 0.32 µm, it decreased during further hydrolysis to 1.79 ± 0.18 µm (SI, Fig. S5), which could result from reduced aggregation due to increased solubility. The particle size of sodium caseinate was constant (0.25 ± 0.03 µm) until a DH of 10.6% was reached. The average particle size can be masked by the overall distribution of particles and the peptide interactions in a sample at a different DH. The average particle size of sodium caseinate hydrolysates at a higher DH (DH > 11.9%) increased to 0.56 ± 0.25 µm (SI, Fig. S5), probably due to the intermolecular interaction of the molecules. The average particle size of hydrolyzed buffalo worm protein decreased from an initial 3.83 ± 0.56 µm to 2.16 ± 0.25 µm at a DH of 11.0% and was constant at a higher DH (SI, Fig S5), which could be a result of increased solubility and less aggregation.

Additionally, the viscosity of the food protein solutions/dispersions was modified during the hydrolysis process (Table 1). Sodium caseinate, which had a high dynamic viscosity of 43.1 ± 0.1 mPa*s, showed a decreased viscosity of 2.3 ± 0.1 mPa*s (– 95%) after hydrolysis (DH = 13.1%). The high dynamic viscosity is caused by the poor tertiary structure of the native sodium caseinate. The peptides released interact less with each other during hydrolysis.

The dynamic viscosity of wheat gluten could not be determined due to its insoluble character. Here, the viscosity depended on the shear time. The viscosity of unhydrolyzed wheat gluten increased from 4.3 ± 0.8 mPa*s (0–10 min shear time) to 11.9 ± 0.6 mPa*s after 30 min shear time (SI, Fig. S6). This may be caused by the cross-linking of the fibrillar proteins gliadin and glutenin due to the shear forces applied. The viscosity of wheat gluten decreased during hydrolysis due to reduced peptide size and less cross-linking possibilities of gliadin and glutenin. The dependency of the viscosity to shear time was no longer observed at a DH of 7.6%. Therefore, hydrolysis of the wheat gluten proteins and the subsequent increase in solubility caused a Newton-like behavior of the fluid. A constant viscosity of 3.2 ± 0.0 mPa*s was detected at the end of hydrolysis (DH = 15.3%). In the case of buffalo worm protein, the dynamic viscosity slightly decreased from an initial 1.7 ± 0.1 to 1.5 ± 0.1 mPa*s (– 11.8%) at the end of hydrolysis (DH = 20.7%), which is in line with the increased solubility and decreased particle size.

Theoretical bitter potential of the hydrolysates

The theoretical bitter potential was determined according to Ney [47] after mass spectrometric analysis of peptides formed. Peptides with a Q-value > 1400 and a molecular size < 6 kDa show a high potential of expressing a bitter taste [47]. Peptides with a bitter potential seem to be distributed throughout all casein fractions, with a maximum number (59%) found in β-casein (SI, Table S2). During hydrolysis, the percentage of potentially bitter peptides in α_{S1}-casein increased to 46%, while it remained constant in α_{S2}-casein (~56%) and β-casein (~60%). In contrast, κ-casein showed a decrease to 48%. These findings suggest that the mixture of peptides from different casein fractions helps balance overall bitterness.

Most peptides (10–21) expressing a bitter potential in wheat gluten hydrolysates were found in the glutenin fractions. However, the percentage of bitter peptides remained generally constant across wheat gluten fractions (~38% in α/β-gliadin, ~24% in γ-gliadin, and ~10% in glutenin). For γ-gliadin, the percentage increased to 67% at a DH of 11.9%, but then decreased to 24% at a DH of 15.3%. This suggests that bitter peptides were initially solubilized during hydrolysis and then further broken down, reducing their bitterness. Buffalo worm protein hydrolysates exhibited a low and consistent percentage of potentially bitter peptides (10–12%) throughout hydrolysis. The reduced bitterness in both wheat gluten and buffalo worm protein hydrolysates is likely linked to the removal of insoluble protein fractions during sample preparation. However, the potential for bitterness also depends on the concentration and interaction of these peptides, so these results should be considered an estimation of potential bitterness in the hydrolysates.

Emulsifying properties of the hydrolysates

Intact proteins may not or only partially interact with oil–water interfaces due to their size and conformation, limited solubility or lack of exposed hydrophobic areas. The enzymatic hydrolysis of proteins can alter these characteristics and, therefore, their technofunctionality at oil–water interfaces.

Interfacial behavior of the peptides

The interfacial tension of the hydrolysates gives a first impression about the ability of the proteins and peptides to adsorb at and stabilize oil–water interfaces (Table 2, SI, Fig. S7). The interfacial tension of water was 25.3 ± 0.3 mN/m. By comparison, Tween 20, a known emulsifier, showed an interfacial tension of 7.8 ± 0.1 mN/m, which is suitable for the adsorption of the interface and subsequent stabilization of emulsions. The interfacial tension of the unhydrolyzed food proteins was in the respective order: sodium caseinate (13.6 ± 0.2 mN/m) < wheat gluten (14.9 ± 0.3 mN/m) < buffalo worm protein (17.8 ± 0.1 mN/m). The interfacial tension of sodium caseinate increased slightly during hydrolysis (15.3 ± 0.1 mN/m), indicating a modified behavior of the peptides at the interface. This could be attributed to the formation of smaller peptides, resulting in a reduced exposure of hydrophobic areas on the peptide surface, which correlates with the decreased surface hydrophobicity of the hydrolysates. The increased interfacial tension of unhydrolyzed gluten compared to sodium caseinate is caused by the poor solubility of wheat gluten in water systems. The solubility of the proteins increased during the hydrolysis of wheat gluten. Consequently, the interfacial tension of wheat gluten decreased to 12.1 ± 0.1 mN/m, indicating the improved ability of the proteins to adsorb at the interface. The interfacial tension of buffalo worm protein increased slightly during hydrolysis (18.3 ± 0.1 mN/m). The effect of the peptides released was probably masked by the mixture of macromolecules, for example, insoluble carbohydrates and proteins. The emulsion half-life and the oil-droplet size were investigated further for a full evaluation of the emulsion stability.

Model emulsions of the food protein hydrolysates

Oil-in-water emulsions were produced with Mygliol 812N® (20% (w/w)) to investigate the emulsifying properties of the hydrolysates. The mean diameter $d_{4/3}$ of oil droplets in sodium caseinate emulsions was 25.2 ± 0.8 µm (Table 2, Fig. 2A) before decreasing to 19.2 ± 1.3 µm (DH = 5.6%) and then increasing to a maximum of 27.7 ± 0.9 µm (DH = 13.1%) (Fig. 2A). For wheat gluten emulsions, the

Table 2 Emulsifying properties of sodium caseinate, wheat gluten and buffalo worm protein hydrolysates at different degrees of hydrolysis generated by AprX from *P. paralactis*

Protein	Sodium caseinate				Wheat gluten				Buffalo worm protein			
DH [%]	0	8.97	10.6	13.1	0	9.6	11.9	15.3	0	8.7	14.2	20.6
Final interfacial tension [mN/m]	13.6 ± 0.2	14.8 ± 0.4	15.3 ± 0.1	18.4 ± 0.6	14.9 ± 0.3	11.6 ± 0.1	12.1 ± 0.1	12.1 ± 0.1	17.8 ± 0.1	16.6 ± 0.7	18.3 ± 0.1	18.5 ± 0.8
Oil droplet size [µm]	25.2 ± 0.8	21.0 ± 1.6	23.9 ± 1.8	27.7 ± 0.9	51.5 ± 4.7	37.1 ± 2.5	35.0 ± 1.9	37.5 ± 3.3	36.8 ± 4.0	31.2 ± 3.0	30.6 ± 2.5	36.4 ± 3.2
ζ-Potential [mV]	−69.2 ± 2.5	−64.5 ± 2.0	−61.1 ± 1.7	−56.4 ± 4.3	−15.6 ± 1.9	−22.1 ± 1.4	−27.8 ± 1.6	−31.0 ± 1.7	−41.7 ± 3.1	−43.2 ± 4.0	−40.4 ± 3.9	−41.2 ± 3.2
Half-life $t_{1/2}$ [min] of emulsion	23 ± 10	150 ± 0	180 ± 0	7 ± 2	1.5 ± 0.5	16 ± 3	45 ± 5	11 ± 2	45 ± 5	65 ± 5	85 ± 5	57 ± 9

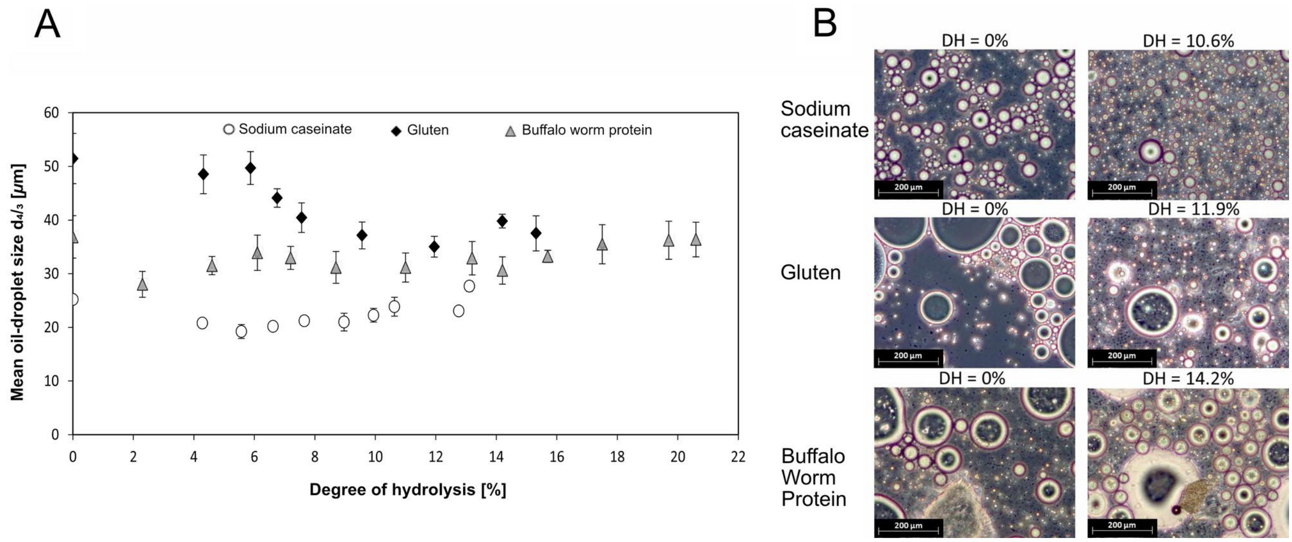

Fig. 2 **A** Oil-droplet size and **B** microscopic images of emulsions prepared with protein hydrolysates from sodium caseinate, wheat gluten and buffalo worm protein generated by AprX from *P. paralactis* (80% continuous phase: hydrolysate, 20% dispersed phase: Mygliol 812 N®, double triplicate measurement; B: magnification 10x)

mean diameter was constant at 51.5 ± 4.7 µm up to a DH of 5.9%, then decreased to 35.0 ± 1.9 µm (DH = 11.9%) (Fig. 2A). The mean diameter of oil droplets in emulsions made with buffalo worm protein hydrolysates decreased from an initial 36.4 ± 4.0 µm to 28.0 ± 2.4 µm (DH = 2.3%), before rising to 33.9 ± 3.3 µm (DH = 6.1%) and seemed constant at a higher DH (Fig. 2A). Thus, no major changes were observed in the mean diameter of oil droplets. Additionally, the span was calculated for the results obtained for more insights. Maximum span was determined for the DH of 14.2% (1.11), indicating a broader range of particle populations induced by hydrolysis via AprX (span of native buffalo worm protein emulsions: 1.01). In addition, a slight widening towards smaller oil droplet sizes was observed. Smaller oil droplets generally improve emulsion stability, and the mean diameter of the oil droplets was lower or similar compared to the initial protein, indicating better (sodium caseinate and wheat gluten) or similar (buffalo worm protein) interface stabilization. However, the increase in droplet size at the end of sodium caseinate hydrolysis suggests reduced interaction with the interface.

Microscopic images revealed that emulsions with unhydrolyzed proteins had loosely packed structures with hollow spaces, characteristic for unstable emulsions (Fig. 2B). Unhydrolyzed wheat gluten and buffalo worm protein emulsions seemingly displayed non-homogeneous oil droplet populations, including large droplets indicating instability (Fig. 2). Emulsions with sodium caseinate appeared more homogeneous. After hydrolysis with AprX, the oil droplets formed in the emulsion seemed more tightly packed, as indicated by narrower spaces in between (Fig. 2B, sodium caseinate / wheat gluten) suggesting improved emulsion stability. At maximum DH, the oil droplet size distribution seems to increase, accompanied by a loosening of droplet packing. Emulsions prepared with buffalo worm protein seemed to show similar oil droplet populations with a slight widening towards smaller oil droplets and contained insoluble cell fragments, likely chitin, observable under the microscope (Fig. 2B).

The surface charge of the oil droplets influences the emulsion properties. A ζ-potential from 0 to ± 30 mV generally indicates emulsion instability, while a ζ-potential greater than + 30 mV or less than – 30 mV suggests instability [54]. The initial ζ-potential of the sodium caseinate emulsions was – 69.2 ± 2.5 mV, indicating a stable emulsion (Table 2). The ζ-potential remained stable initially during hydrolysis and decreased to – 56.4 ± 4.3 mV at the maximum DH. The ζ-potential of wheat gluten emulsions was – 15.6 ± 1.9 mV, suggesting high instability due to poor solubility first, but improved to – 31.0 ± 1.7 mV with increasing DH, implying better solubility and stability (Table 2). The ζ-potential of buffalo worm protein emulsions remained constant between – 40.4 ± 3.9 mV and – 45.6 ± 4.1 mV, indicating stable emulsions across all DH levels, even though larger oil droplet sizes compared to sodium caseinate emulsions were obtained. A stable ζ-potential might be obtained due to the mixture of soluble and insoluble compounds, which could mask charges or evenly distribute electrostatic charges.

Finally, the half-life of the emulsion was determined to correlate these results with the emulsion stability (Fig. 3). Sodium caseinate showed an emulsion half-life of 23 ± 10 min prior to hydrolysis with AprX. The half-life was increased during hydrolysis and reached the maximum value of 180 ± 0 min at a DH of 10.6%, before decreasing to

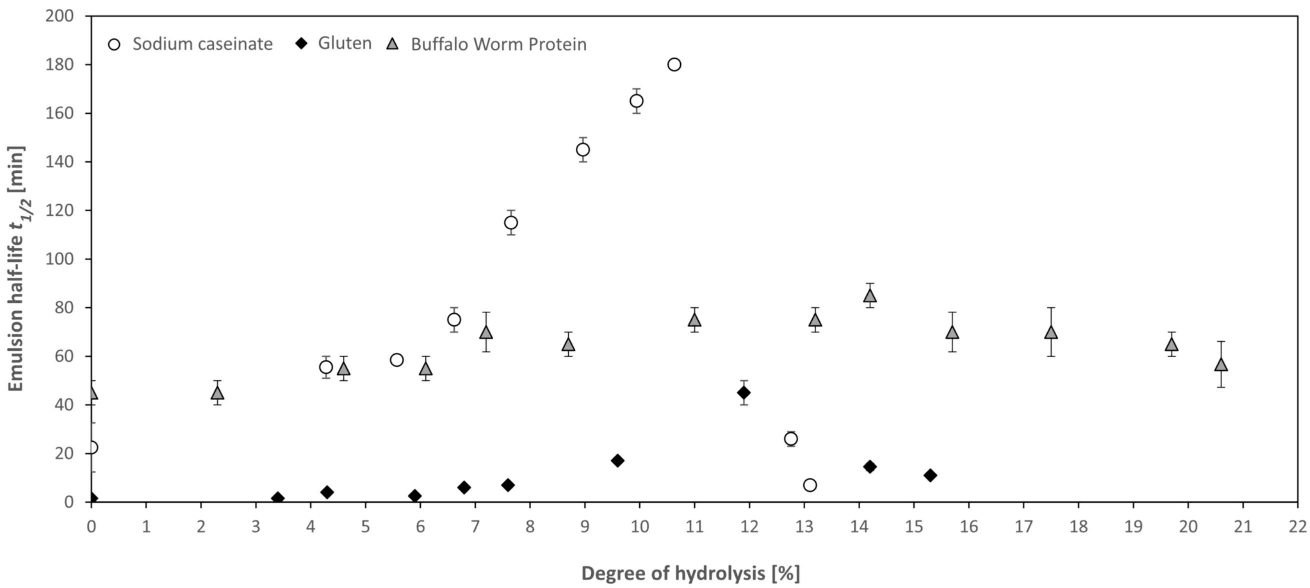

Fig. 3 Half-life of emulsions prepared with sodium caseinate, wheat gluten and buffalo worm protein hydrolysates at each DH of Fig. 1 (continuous phase: 80% (w/w) H_2O_d with 0.9% (w/v) protein, dispersed phase: 20% (w/w) Mygliol 812 N®). Hydrolysates generated by hydrolysis using AprX from *P. paralactis*

7 ± 1 min at the end of the hydrolysis (DH = 13.1%) likely attributed to the generation of peptides with too little amphiphilic character. The emulsion half-life of wheat gluten and its hydrolysates increased from an initial 1.5 ± 0.5 min to a maximum of 45 ± 5 min (DH = 11.9%). The increase of the emulsion half-life is attributed to the increase of the solubility after the hydrolysis and the presence of more soluble peptides, which can stabilize the oil–water interface. Unhydrolyzed wheat gluten contains several insoluble proteins that tend to precipitate, leading to an instable emulsion. The initial half-life of emulsions produced with buffalo worm protein was 45 ± 5 min. The half-life increased to 85 ± 5 min at a DH of 14.2% due to the release of more amphiphilic peptides that stabilize the emulsion indicated by a higher GRAVY and solubility compared to the untreated protein. However, after reaching maximum stability for all food protein hydrolysates, the emulsion half-life decreased with further increases in DH. This decline is attributed to the presence of small peptides that are unable to adsorb effectively at the oil-in-water interface.

Discussion

Hydrolysis of proteins and emulsion stability

In this study, three food proteins of different origins were hydrolyzed using AprX from *P. paralactis*. Originally identified in raw milk and associated with milk quality loss [31, 55], *Pseudomonas* AprX has broad substrate selectivity [32, 33], making it ideal for investigating food protein hydrolysis. AprX is classified as a metallopeptidase stabilized by Ca^{2+} [33] and showed improved stability during the hydrolysis of sodium caseinate, which is naturally rich in Ca^{2+} (150–400 mg/100 g) [56]. The lower Ca^{2+} content in wheat gluten (90–150 mg/100 g) [57] and insect protein (44–64 mg/100 g) [58] may explain the reduced residual activity. Despite this, the highest DH was observed in buffalo worm protein hydrolysates, with similar DH values found in wheat gluten and sodium caseinate hydrolysates. The differences in DH are likely due to variations in amino acid sequences and accessible cleavage sites in the proteins.

This study is the first to utilize AprX, typically associated with milk destabilization, for the tailored hydrolysis of food proteins intended for emulsification. The gene sequence of AprX from *P. paralactis* can be transferred to a microbial production strain established in the food industry to ensure food grade production in the future. The hydrolysis results were compared with those using commercial enzymes (Table 3). A similar hydrolysis process was performed on sodium caseinate using the commercial enzyme Sternzym BP 25201, a thermolysin-like peptidase from *Geobacillus stearothermophilus* (activity 15 nkat mL^{-1}), which is more selective towards hydrophobic amino acids (i.e., Leu, Phe, Ile, Val at P1‘) [41]. The maximum half-life reported was 125 ± 12 min at a DH of 2.2%, with a fivefold increase in half-life. AprX achieved a DH of 10.6% with increased emulsion stability, attributed to its broader substrate selectivity and more extensive protein cleavage. No supramolecular structures were observed in this study, suggesting a different stabilization mechanism.

Table 3 Influence of enzymatic hydrolysis on emulsion stability of sodium caseinate, gluten and buffalo worm protein using different peptidases

Substrate	Peptidase preparation	DH [%]	Initial		Maximum		Increase of emulsion stability [n-fold]	References
			ESI [min]	$t_{1/2}$ [min]	ESI [min]	$t_{1/2}$ [min]		
Sodium caseinate	AprX	10.6	36 ± 3	23 ± 10	337 ± 6	180 ± 0	8*	This work
	Sternzym BP 25201	3.8	n.a	25 ± 3	n.a	125 ± 12	5	Ewert *et al.* [45]
	Papain	13.3	34 ± 6	n.a	93 ± 9	n.a	3	Luo *et al.* [59]
	Trypsin	20.7	34 ± 6	n.a	66 ± 10	n.a	2	
	Pancreatin	9.4	34 ± 6	n.a	62 ± 10	n.a	2	
Wheat gluten	AprX	11.9	11 ± 0	1.5 ± 0.5	226 ± 3	45 ± 5	30*	This work
	Protamex™	2.8	9 ± 2	n.a	38 ± 1	n.a	4	Wang *et al.* [61]
	Alcalase 2.4L	5.0	n.a	n.a	n.a	n.a	2	Kong *et al.* [62]
	Acidic protease	4.9	6 ± 0	n.a	6 ± 0	n.a	0	Deng *et al.* [9]
Buffalo worm protein	AprX	14.2	54 ± 2	45 ± 5	152 ± 9	85 ± 5	2*	This work
Cricket	Alcalase 2.4L	52.4 ± 0.8	< 30	n.a	~ 50	n.a	1.5—2	Hall *et al.* [65]

ESI = emulsion stability index, $t_{1/2}$ = emulsion half-life, *n.a.* = not available

*Calculation based on $t_{1/2}$

Other peptidases reported like papain, trypsin, and pancreatin for sodium caseinate hydrolysis, have a narrower enzyme selectivity compared to AprX and result in an 2–threefold increase in emulsion stability despite similar DH ranges reached [28, 59, 60]. The broader substrate selectivity of AprX likely enhances peptide amphiphilicity, contributing to better stabilization at the interface.

For wheat gluten hydrolysates, enzyme preparations such as ProtamexTM, Alcalase 2.4L, and acidic peptidases, which target hydrophobic residues, showed up to a fourfold increase in emulsion stability [9, 61–63]. Our study achieved a 30-fold increase in emulsion stability using AprX, surpassing previous reports by 7.5 times. Unlike most studies on insect protein hydrolysis, which focus on bioactivity and other physiological properties [14], we compared our results with studies on DH and technofunctional properties. For example, cricket (*Gryllodes sigillatus*) protein hydrolysis using Alcalase 2.4L resulted in a DH between 15 and 85%, depending on enzyme ratio and hydrolysis time [64]. A different study reported emulsion stability indices of around 50% after 60 min of hydrolysis [65]. Similarly, *Bombyx mori* chrysalis protein hydrolysis using six enzymes achieved the highest DH (> 30%) with Alcalase 2.4L, aligning with our findings [66]. Hydrolysis of cricket (*Acheta domesticus*) and mealworm (*Tenebrio molitor*) proteins using ProteaseA and Flavourzyme 1000L yielded in DHs of 46% and 33% for cricket, and 71% and 51% for mealworm, respectively, likely due to the synergistic effects of exo- and endopeptidase activities [67]. Overall, the technofunctional properties are highly dependent on the enzyme used and the hydrolysis conditions, making direct comparisons only partially meaningful..

Bitterness of the hydrolysates

Bitter peptides in dairy products have been extensively investigated in the past, especially when released from casein fractions by hydrolysis via cell-envelope peptidase from *Lactococcus* sp. [48, 49]. Four of the peptides identified as bitter peptides in dairy products previously [48, 49] were found in our study released by AprX from sodium caseinate: FFVAPFPEVF (α_{S1}-casein$_{23-32}$), VENLHLPLPLL (β-casein$_{130-140}$), LPLPLLQSW (β-casein$_{135-143}$) and FFSDKIAK (κ-casein$_{17-24}$). Based on this, it is likely that the sodium caseinate hydrolysates produced by AprX from *P. paralactis* express a bitter taste. However, no such study was found for the other substrates–wheat gluten and buffalo worm protein–used in this study. Based on the evaluation of the bitter potential according to Ney [47], it seems that bitter hydrolysates from wheat gluten were released while the proteins were solubilized. Although the theoretical bitter potential according to Ney [47] remained constant for buffalo worm protein hydrolysates, hydrolysates might be perceived with increasing bitterness, as is described for other insect protein sources, such as lesser mealworm or cricket protein [67]. However, since emulsifiers are applied in small amounts and in combination with strong tasting ingredients, the bitter potential might be masked in the final product [45].

Proposed mechanisms for the stabilization of emulsions by food protein hydrolysates

The stabilization of emulsions by protein hydrolysates is assembled by the synergy of micromolecular and macromolecular parameters. Micromolecular parameters are the particle size, the peptide composition, viscosity, solubility and surface hydrophobicity. Macromolecular parameters are described by the oil-droplet size, ζ-potential and the emulsion stability [68, 69]. Overall, three different mechanisms are proposed for the proteins explored after hydrolysis with AprX from *P. paralactis* (Fig. 4).

Sodium caseinate hydrolysates

Unhydrolyzed sodium caseinate can adsorb loosely at the oil–water interface without forming a network [70]. AprX-induced hydrolysis of sodium caseinate releases small peptides (10–15 kDa) with amphiphilic properties, facilitating faster interface adsorption [2]. These peptides exhibit an enhanced potential for protein–protein interaction, stabilizing the emulsion through intermolecular interactions [71]. The resulting lamella at the interface enhances steric stabilization [2]. Surface hydrophobicity results suggest that hydrophobic structures of the peptides are not exposed, rendering them more flexible at the interface and causing a reorientation of internal hydrophobic structures [72]. On a macroscopic level, the emulsion stability increases primarily due to the reduced oil-droplet size. However, at a DH > 10.6%, emulsion stability decreases, as peptides become too small to express amphiphilic characteristics, limiting the interaction with the interface.

Wheat gluten hydrolysates

Unhydrolyzed wheat gluten is colloidally dissolved through the formation of a network by glutenins and gliadins, primarily held together by non-covalent interactions and disulfide bridges [11, 73]. This structure limits interactions at the oil–water interfaces. AprX-induced hydrolysis modifies the network, yielding small peptides with an enhanced solubility and technofunctionality. The increased solubility is probably the driver for the improved technofunctionality: The solubilized peptides, exposing hydrophobic regions at the surface, can diffuse to the oil–water interface with a higher adsorption rate compared to the native protein, increasing their amphiphilic character of the interface [2]. On a macroscopic level, the emulsion stability improves due to the increased surfactant activity of peptides (i.e., faster stabilization of the oil–water interface) and consequently, reduced oil-droplet size. Furthermore, the ζ-potential decreases, causing an enhanced electrostatic repulsion of oil droplets. However, further hydrolysis (DH > 11.9%) results in the production of too small peptides that cannot adsorb at the interface.

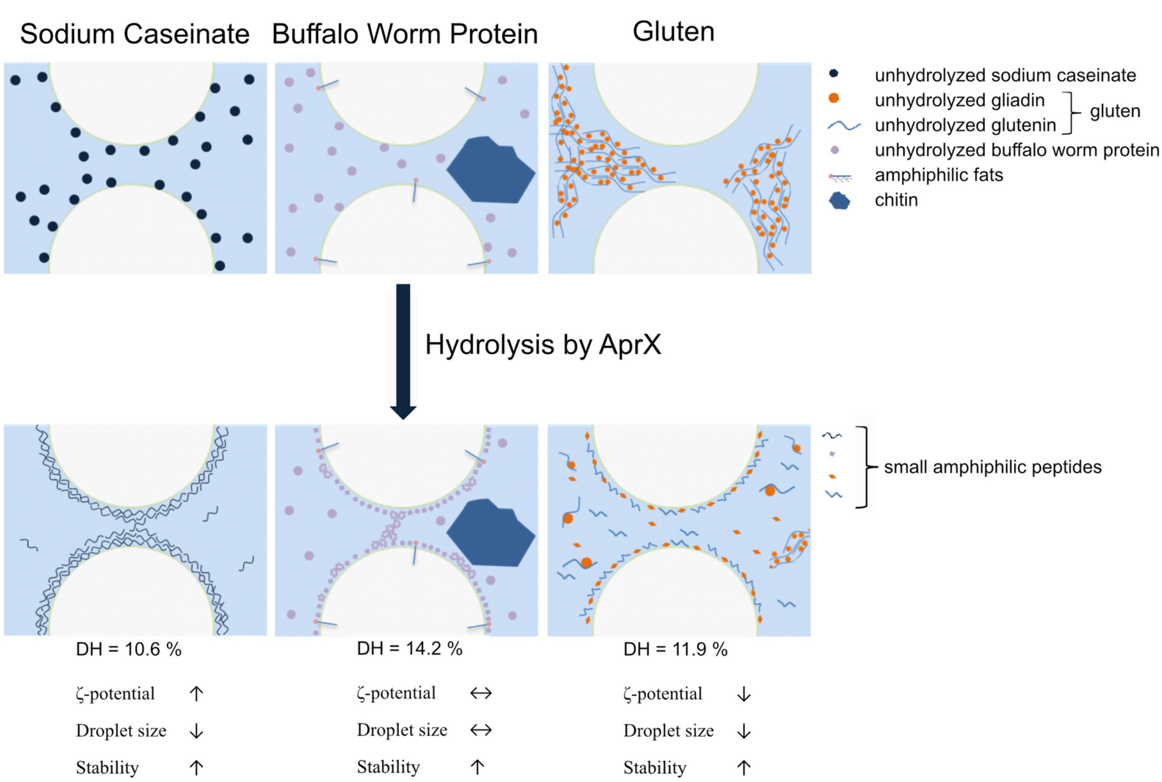

Fig. 4 Proposed mechanisms of interaction of sodium caseinate, wheat gluten and buffalo worm protein hydrolysates at the oil–water interface. Hydrolysates generated by hydrolysis using AprX from *P. paralactis*

Buffalo worm protein hydrolysates

The buffalo worm protein used in this study is a complex mixture comprising macromolecules such as proteins, carbohydrates, fibers and residual fats. Removal of major fat content via centrifugation lead to a mixture of soluble and insoluble proteins, carbohydrates and fibers. Since samples were compared with the untreated reference sample and AprX does only contain peptidase activity, improved emulsifying properties are likely attributed to the protein fraction and its hydrolysates. The emulsifying properties are attributed to protein flexibility [74], allowing both globular proteins and amphiphilic peptides generated during hydrolysis by AprX to partially unfold at the interface, leading to a reorientation of hydrophilic and hydrophobic residues. Based on the results obtained here, it can be hypothesized that the emulsion stabilization with buffalo worm protein hydrolysates is a unique combination of effects, driven by the decreased particle size, increased protein solubility, and an increased amphiphilicity (increased GRAVY) of the peptides. As neither the ζ-potential, nor the oil-droplet size have been majorly impacted by the hydrolyzed buffalo worm protein, the increased stabilization seems more due to tighter coverage of interface and increased hydrophobicity, in contrast to a faster peptide absorption or charge driven stabilization. The prolonged hydrolysis time (DH > 14.6%) diminishes the emulsion stability, due primarily to the formation of small peptides and their agglomeration, similar to observations in sodium caseinate.

Conclusion

In summary, the endopeptidase AprX from *P. paralactis* originating from raw milk proved effective in generating functional peptides during hydrolysis. Its broad selectivity resulted in high degrees of hydrolysis for sodium caseinate, wheat gluten and buffalo worm protein. This study revealed that hydrolysates with DH up to 10.6–14.2% altered the emulsifying properties positively. The combination of a high DH with improved solubility and technofunctionality holds great promise for the food industry, offering natural protein-based emulsifiers applicable in diverse products, for example, beverages, creamers, culinary solutions and high protein formulations.

Despite concerns about the potential bitterness, a minimal impact due to the low emulsifier concentration and reduced bitterness after interface interaction is expected. This bitterness can also be masked by synergies with strong tasting ingredients, such as sugars or salts. Additionally, a strategy involving endopeptidase hydrolysis, followed by exopeptidase treatment, as demonstrated by Ewert *et al*. [27], may mitigate the bitterness, while maintaining enhanced functionality. Further research should explore these aspects, contributing to the development of advanced and palatable food ingredients.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-024-04644-3.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human or living animal subjects.

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