Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: Partial characterizations

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ABSTRACT

Sodium caseinate (NaCas) was hydrolyzed by papain, pancreatin and trypsin from 10 min to 24 h, and the hydrolysates were partially characterized for several important properties. At the studied conditions, papain and trypsin were more effective in hydrolyzing NaCas than pancreatin. Pancreatin treatments showed an initial increase in surface hydrophobicity, contrasting with the consistent decrease for the other two treatments. The solubility of NaCas at acidic pH was improved, becoming pH-independent after 24 h hydrolysis. The emulsifying properties generally showed improvements after hydrolysis. The DPPH free radical scavenging activity, reducing power, and inhibition of linoleic acid autoxidation were significantly enhanced after appropriate hydrolysis, while metal ion chelating effects were slightly attenuated. The angiotensin converting enzyme-inhibitory activity was significantly improved by up to 9 times than that of NaCas. These findings indicate that physical, chemical and biochemical properties of casein hydrolysates can be improved by selecting proteolytic conditions to produce functional ingredients.

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1. Introduction

Protein hydrolysates have attracted increasing attention since last decade because of the enhancement in functional properties and health-promoting bioactivities from their precursor proteins. The functionalities of particular interest include the improved solubility, particularly at pH near the isoelectric point (pI), enhanced emulsifying properties, as well as enriched biological activities. Especially, due to bioactive peptides produced during enzymatic hydrolysis, the health-promoting effects of hydrolysates, such as antihypertensive, antithrombotic, anticancer, immunomodulatory and opioid activities have been extensively reported (Udenigwe & Aluko, 2012).

Caseins are a group of soluble milk proteins and are present as casein micelles consisting of \( \alpha s1-\), \( \alpha s2-\), \( \beta-\) and \( \kappa-\) caseins, and the ratio of the four caseins differs in mammals. Enzymatic hydrolysis of caseins has been reported to have improved functional properties and bioactivities, with the extent depending on the protease type and hydrolysis time (López-Fandiño, Orte, & van Camp, 2006). For instance, casein is well-known for its strong mineral-binding capability, but the poor solubility of casein-mineral complexes at gastrointestinal conditions results in low bioavailability of the minerals (Korhonen, 2009). An improvement in the bioavailability of zinc has been reported after forming complexes with enzymatic hydrolysates of yak casein (Wang, Zhou, Tong, & Mao, 2011). The health-promoting benefits of yak casein, such as free radical scavenging capacity and anti-inflammatory activity, were also dramatically enhanced by proteolysis, with alcalase being the most effective enzyme, followed by trypsin (Mao, Cheng, Wang, & Wu, 2011). Similar improvements in health benefits were reported after the enzymatic hydrolysis of camel casein (Salami et al., 2010) and ovine casein (Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, & Recio, 2008).

Bovine milk is the most commonly consumed milk and is processed to various dairy ingredients and products. Sodium caseinate (NaCas) is a dairy ingredient developed to enhance functionalities such as water solubility, emulsifying and foaming properties, as well as encapsulation capabilities (Pan, Zhong, & Baek, 2013; Sánchez & Patino, 2005). NaCas has also been recently explored as a novel stabilizer and absorption enhancer of hydrophobic protein nanoparticles (Luo, Teng, Wang, & Wang, 2013; Zhang et al., 2014). However, the solubility of NaCas at acidic pHs near the pI (around pH 4.6) is poor, and its biological activities are limited. Enzymatic hydrolysis has recently been shown to enhance the bioactivities of NaCas, including inhibition of angiotensin I-converting enzyme (ACE), antimicrobial properties and antioxidant capabilities (Chen & Li, 2012; Hogan, Zhang, Li, Wang, & Zhou, 2009).

Despite numerous studies on the properties of casein hydrolysates, the impact of proteolytic conditions on important physical,
chemical and biochemical properties and the correlation to structural characteristics is needed. The first objective of this work was to compare the proteolytic kinetics of NaCas by papain, trypsin and pancreatin, partially characterized by the degree of hydrolysis (DH) and gel electrophoresis. The second objective was to characterize physical, chemical and biochemical properties of the obtained hydrolysates. These properties included solubility, surface hydrophobicity, emulsifying properties, antioxidant capacities and ACE-inhibitory activities. The information may be used by the industry to produce hydrolysate ingredients with desired properties. Although not attempted in the current paper, findings from this work may be used to select proteolytic conditions in order to produce hydrolysates that could afford peptides with bioactivities and the analysis of their amino acid sequences.

2. Materials and methods

2.1. Materials

NaCas, pancreatin (catalogue number P1750) and trypsin from porcine pancreas (catalogue number T0303, 13,000–20,000 BAE units/mg protein), ACE (from rabbit lung), substrate peptide (hippuryl-histidyl-leucine, HHL), and 8-anilinonaphthalene-1-sulfonic acid (ANS) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Papain (catalogue number AC41676, 46,000–50,000 nfu/mg protein) was obtained from Acros Organics (Morris Plains, NJ, USA). Bovine serum albumin (BSA) used in the protein assay was from BioWorld LLC (Atlanta, GA, USA). Unless noted otherwise, other chemicals were of analytical grade and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of hydrolysates

To prepare hydrolysates, NaCas was dissolved at 5% w/v in 250 ml of 0.01 M phosphate buffer saline (PBS) and the enzyme was dissolved at an enzyme: substrate mass ratio of 0.5:100. The pH was adjusted to 7.0 for papain treatments and 8.0 for pancreatin and trypsin treatments. Samples were then incubated in a water bath shaker (C76 classic series, New Brunswick Scientific, NJ, USA), with temperature and speed set as 37 °C and 300 rpm, respectively. Samples (35 ml) were taken after hydrolysis at 10, 30 min, 1, 4 and 24 h, without pH adjustment during hydrolysis. After inactivating enzymes by boiling for 10 min, the samples were centrifuged (SORVALL RC5B Plus centrifuge, DuPont, Wilmington, DE, USA) at 5000 g for 20 min to remove any insoluble contents. The supernatants were collected, freeze-dried (VirTis AdVantage Plus EL-85benchtop freeze dryer, SP Scientific Inc., Gardiner, NY, USA), and stored at −20 °C for further use. The protein concentration in each hydrolysate was determined using the bicinchoninic acid (BCA) assay kit, with BSA as a protein standard. The hydrolysates were incubated at 21 °C for 2 h. The absorbance at 562 nm was measured using a UV/vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA, USA).

2.3. Degree of hydrolysis (DH)

The DH of hydrolysates was determined by reacting free amino acids with o-phthalaldehyde (OPA), according to a reported method (Nielsen, Petersen, & Dambmann, 2001), with modifications. Briefly, 50 μl of a test sample was mixed with 3 ml of an OPA working reagent that was freshly prepared to a total volume of 200 ml by mixing the following solutions: 25 ml of 100 mM sodium borate aqueous solution, 2.5 ml of 20% sodium dodecyl sulphate aqueous solution, 0.16 g of OPA in 4 ml ethanol, 400 μl of β-mercaptoethanol and the remainder being distilled water. After vortexing for 5 s and incubation at room temperature (21 °C) for 2 min, the absorbance of the mixture (A_sample) was measured at 340 nm (Evolution 201, Thermo Scientific, Waltham, MA, USA). To determine the highest possible DH, NaCas was hydrolysed in 6 N HCl for 24 h at 120 °C (Chen et al., 2013), and the product obtained was reacted with OPA as described above to obtain its absorbance (A_total) as an indicator of the maximum DH. The DH in enzymatic hydrolysates was then calculated by the following equation:

\[
DH\% = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{total}} - A_{\text{blank}}} \times 100
\]

where A_blank is the absorbance of the blank prepared by replacing the test sample with distilled water in the above assay.

2.4. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The reducing SDS–PAGE was performed using a precast 15% gradient polyacrylamide gel. The test samples were diluted 10 times in an SDS–PAGE sample buffer containing β-mercaptoethanol (Bio-Rad Laboratories Inc., Hercules, CA, USA) and were heated at 95 °C for 5 min. Ten μl of samples with 4 mg/ml protein was loaded onto the gel for electrophoresis at 200 V. After staining using Coomassie brilliant blue G-250 and destaining overnight, the gel was scanned. The Precision Plus Protein™ standard (Bio-Rad, Hercules, CA, USA) was used as molecular weight marker.

2.5. Surface hydrophobicity (S₀)

The S₀ was determined using fluorescence probe ANS, according to a literature method (Wu, Hettiarachchy, & Qi, 1998), with minor modifications. Briefly, the freeze-dried hydrolysate or NaCas powder was dispersed in 0.01 M PBS (pH 7.0) at 1 mg/ml as stock solutions, which were then diluted with PBS to a protein concentrations range of 5–100 μg/ml. The diluted samples (4 ml) were added to a test tube and mixed with 20 μl of an ANS working solution that was previously prepared at 8 mM in 0.01 M PBS (pH 7.0). The tubes were incubated for 2 h at room temperature (21 °C) in the dark. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 507 nm using a fluorescent spectrophotometer (model RF-1501, Shimadzu Corp., Tokyo, Japan). The initial slope of fluorescent intensity vs. sample concentration plots after linear regression with R² > 0.995 was used as an index for S₀.

2.6. Solubility measurement

The solubility of NaCas and its hydrolysates was measured as a function of pH. The freeze-dried sample was dispersed in distilled water at 10 mg/ml, and the pH was adjusted to 3–9 with 1 N HCl or NaOH. After incubation at different pHs for 30 min at room temperature, samples were centrifuged at 14,100 g for 10 min (model 4540 R, Eppendorf, Hamburg, Germany). The protein content in the supernatant was measured by the BCA method, as detailed above. The solubility was calculated as the percentage of the protein concentration to that of the corresponding solution at pH 9 (Kasran, Cui, & Goff, 2013).

2.7. Emulsifying activity and emulsion stability indexes

Emulsifying activity (EAI) and emulsion stability indexes (ESI) of hydrolysates were determined by the turbidimetric method (Pearce & Kinsella, 1978), with slight modifications. Thyme oil was used as model oil. Briefly, 3 ml of thyme oil and 9 ml of 0.1% w/v hydrolysate solution (in 0.01 M PBS, pH 7.0) were mixed and homogenized with a Cyclone 1Q, microprocessor homogenizer.
(VirTis, Gardiner, NY, USA) at 12,500 rpm for 1 min. A 30 μl sample of the prepared emulsion was withdrawn at 0 and 10 min after homogenisation, followed by diluting 500 times with 0.1% SDS solution. Then, the absorbance was measured at 500 nm using the above UV–vis spectrophotometer. The EAI and ESI were calculated according to Eqs. (2) and (3), respectively.

\[
\text{EAI (m²/g) = } \frac{2T \times A_0 \times \text{dilution factor}}{C \times \phi \times 10,000} \quad (2)
\]

where \(T\) is a constant (2.303 m²/mJ), the dilution factor was 500, \(C\) is the protein concentration in the aqueous phase before emulsion preparation (g/ml), \(\phi\) is the volumetric fraction of oil (0.25), and \(A_0\) is the absorbance right after homogenisation (0 min).

\[
\text{ESI (min)} = \frac{A_0 - \Delta A}{\Delta t} \quad (3)
\]

where \(\Delta A\) is the absorbance decrease from \(A_0\) after \(\Delta t\) (10 min).

2.8. Antioxidant properties

2.8.1. Free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of hydrolysates and NaCas were determined according to the method described by Zhang, Luo, and Wang (2011), with some modifications. Hydrolysates were prepared at 2 mg/ml in distilled water and 1 ml of a sample was mixed with 2 ml of 0.1 mM DPPH dissolved in ethanol. The mixture was vortexed and allowed to stand in the dark at room temperature (21 °C) for up to 24 h, and the absorbance was measured at 517 nm during storage (Evolution 201, Thermo Scientific, Waltham, MA, USA). The control was prepared by replacing the sample with distilled water. The DPPH radical scavenging was expressed as the percentage as below:

\[
\text{Scavenging activity (%) = } \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (4)
\]

where \(A_{\text{sample}}\) and \(A_{\text{control}}\) are the respective absorbance of test sample and control after a specific incubation time.

2.8.2. Reducing power

The reducing power of samples was tested according a previous method (Wu, Chen, & Shiau, 2003), with modifications. Hydrolysates were prepared at 8 mg/ml in distilled water. A 1 ml aliquot of a hydrolysate sample was mixed with 1 ml of 0.2 M PBS (pH 6.6) and 1 ml of 1% potassium ferricyanide (K₃Fe(CN)₆). The mixture was incubated at 50 °C for 20 min, followed by cooling to room temperature. After adding 2.0 ml of 10% trichloroacetic acid, the mixture was centrifuged at 5,000 g for 20 min. The supernatant was transferred and mixed with 0.3 ml of a 0.1% ferric chloride solution. Following incubation for 10 min at room temperature (21 °C), the absorbance of the resulting solution was measured at 700 nm using the above UV–vis spectrophotometer. The extent of absorbance increase was used to indicate the reducing power of a sample. A control was prepared by replacing hydrolysate sample with distilled water and used in calibration.

2.8.3. Chelating activity

The chelating activity of the samples was estimated by the ferrozine method (Gu et al., 2010), with modifications. All reagents were freshly prepared before use. One ml of a sample with a 4.0 mg/ml hydrolysate solution or NaCas was mixed with 1.0 ml distilled water and 50 μl of 2.0 mM FeCl₂, followed by resting at room temperature (21 °C) for 30 s. After adding 0.1 ml of 5 mM ferrozine, vortexing, and incubation at room temperature (21 °C) for 10 min, the absorbance was measured at 562 nm using the above UV–vis spectrophotometer. The control was determined similarly by replacing the protein sample with distilled water. The chelating activity was calculated as follows:

\[
\text{Chelating activity (%) = } \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \quad (5)
\]

where \(A_{\text{sample}}\) and \(A_{\text{control}}\) are the absorbance of test sample and control, respectively.

2.8.4. Inhibition of linoleic acid autoxidation

The inhibitory effect against linoleic acid autoxidation was evaluated by the ferric thiocyanate method as reported previously (Wu et al., 2003), with modifications. A 2 ml sample with 3 mg/ml hydrolysate or NaCas was mixed with 0.5 ml of 0.1 M PBS (pH 7.0) and 2.0 ml of 50 mM linoleic acid dissolved in ethanol. The mixture was incubated in the dark at 60 °C to accelerate autoxidation. A 50 μl aliquot of the reaction mixture was withdrawn daily and mixed with 2.35 ml of 75% ethanol, 50 μl of 30% ammonium thiocyanate and 50 μl of 20 mM ferrous chloride solution (in 3.5% HCl). The resulting solution was vortexed and incubated at room temperature for 3 min and the absorbance was then measured at 500 nm using the UV–vis spectrophotometer. The number of days to reach the absorbance value of 0.3 was defined as the induction period to indicate the relative antioxidant activity of samples.

2.9. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity was determined by a spectrophotometric method described by Cushman and Cheung (1971), with modifications. A 50 μl sample of a hydrolysate solution (1 mg/ml in PBS, pH 7.0) was mixed with an equal volume of an ACE solution (25 milliunits/ml). Following incubation at 37 °C for 10 min, 150 μl of an HHL substrate solution was added that was previously prepared at 8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3. The mixture was then incubated at 37 °C for 60 min, followed by termination of the reaction by adding 250 μl of 1 N HCl. To extract hippuric acid, 1.4 ml of ethyl acetate was added to the mixture, that was vortexed for 5 s and centrifuged at 14,100g for 5 min. Then, 1 ml of the upper organic phase was transferred into 20 ml glass vials. The ethyl acetate in the glass vials was evaporated at room temperature (21 °C) for 1.5 h in a vacuum oven. After the addition of 2 ml distilled water to dissolve the extracted hippuric acid, the absorbance was measured at 228 nm using the spectrophotometer. The control sample was prepared by replacing the test sample with distilled water. The inhibition (%) was calculated as below:

\[
\text{Inhibition (%) = } \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (6)
\]

where \(A_{\text{control}}\) and \(A_{\text{sample}}\) are the absorbance values of the control and test sample, respectively.

2.10. Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± standard deviation. The one-way analysis of variance (ANOVA) was conducted with Tukey’s multiple-comparison test to compare the significance among samples, using SPSS package (SPSS 13.0 for windows, SPSS Inc, Chicago, IL, USA). The significance level (\(P\)) was set as 0.05.

3. Results and discussion

3.1. Degree of hydrolysis (DH) and SDS–PAGE characterization

The DHs after enzymatic hydrolysis for five different durations are shown in Table 1. The DH was within the range of 9–22% for all
hydrolysates and was significantly higher at longer hydrolysis times. Among the three enzymes, papain was found to be slightly more effective than pancreatin and trypsin at the studied conditions. The DH generally agreed with SDS–PAGE results (Fig. 1). For papain-treated hydrolysates, a smearing zone much smaller than caseins was observed after 10 min hydrolysis and shifted to lower molecular weights (MW) with continued hydrolysis. For pancreatin treatments, several distinct bands with a wide MW distribution were observed until 24 h hydrolysis. Distinct bands were also observed for trypsin treatments after 10 min hydrolysis, and the MWs of bands were much smaller than those of caseins and pancreatin treatments. Papain is a cysteine protease and is an endopeptidase (Nägler et al., 1999), while trypsin is a serine protease with high specificity to C-terminal arginine and lysine residues (Olsen, Eng, & Mann, 2004). Pancreatin is a mixture of enzymes released by the pancreas and has been recently characterized to preferentially cleave N-terminal phosphorylated regions and the C-terminal hydrophobic regions of casein molecules (Su et al., 2012). Differences in the hydrolysis specificity of the proteases agree with the distinct bands (Fig. 1), higher DH (Table 1) of papain treatments and the distinct bands of the other two treatment groups.

### 3.2. Surface hydrophobicity (S₀)

The S₀ of hydrolysates is tabulated in Table 1. For papain- and trypsin-treated hydrolysates, the S₀ decreased gradually with an increase in hydrolysis time, while the S₀ of the pancreatin-treated hydrolysates increased significantly with the increase of hydrolysis time in the first 4 h, before showing a significant decrease after 24 h. The initial increase of S₀ for the pancreatin treatments may be due to the initial production of peptides more hydrophobic than caseins, before excessive hydrolysis to yield low MW hydrophilic peptides occurs. A similar phenomenon has been reported previously, where pancreatic hydrolysis of bovine casein (for a short time) yielded hydrolysates with a more hydrophobic nature, while hydrophilic hydrolysates were obtained after extensive hydrolysis (Su, Qi, He, Yuan, & Zhang, 2007). Papain and pancreatin were found to be the most and least effective enzymes in reducing the S₀, showing the lowest and highest S₀ values, respectively, after 24 h hydrolysis.

### 3.3. Solubility of hydrolysates

The solubility profiles of NaCas at pH 3.0–9.0 before and after hydrolysis are presented in Fig. 2. In the case of NaCas (Fig. 2A), the lowest solubility was observed at pH 4.0, which is near the pI of caseins, and the solubility above pH 4.0 increased with an increase in pH. The solubility of hydrolysates varied with the enzyme type and hydrolysis time. The papain-treated hydrolysates exhibited higher solubility than the other two groups. Papain hydrolysis at only 10 min resulted in an increase in solubility to higher than 65% at pH 4.0, and the solubility became pH-independent and was greater than 90% after 24 h (Fig. 1B). The pancreatin-treated hydrolysates, however, exhibited the lowest increases in solubility at pH 4 and 5 when the time was 4 h or shorter. This trend may be, in part, attributed to the higher MW and S₀ of the pancreatin-treated hydrolysates (Fig. 1) when compared to the other two enzyme treatment groups. After 24 h hydrolysis by pancreatin, the solubility was higher than 80% at the entire pH range. Among the three enzymes, trypsin exhibited the intermediate capability to improve the solubility of hydrolysates with limited hydrolysis durations, and the solubility at pH 4.0, after 24 h hydrolysis, was found to be the lowest, at ca. 70%.

### 3.4. Emulsifying properties

The emulsifying properties at pH 7.0 were evaluated by EAI and ESI (Table 1). When compared to NaCas, both EAI and ESI were significantly different (P<0.05) in the mean within the same enzyme treatment; the uppercase superscript letter A represents the mean is significantly different (P<0.05) from that of control sample, None (NaCas). Data values are expressed as mean ± standard deviation from three replicates.

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>DH (%)</th>
<th>S₀ at pH 7.0</th>
<th>EAI (m²/g)</th>
<th>ESI (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (NaCas)</td>
<td>–</td>
<td>1281.25 ± 106.98</td>
<td>175.64 ± 9.23</td>
<td>33.79 ± 6.03</td>
</tr>
<tr>
<td><strong>Papain treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10 min</td>
<td>13.3 ± 0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>655.00 ± 66.99&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>383.53 ± 16.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.42 ± 9.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 min</td>
<td>17.1 ± 0.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>555.17 ± 42.54&lt;sup&gt;c&lt;/sup&gt;</td>
<td>379.28 ± 21.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.73 ± 9.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 h</td>
<td>16.16 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>337.30 ± 51.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>320.88 ± 35.93&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.26 ± 7.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 h</td>
<td>20.43 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>306.85 ± 38.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>310.46 ± 7.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.14 ± 7.96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h</td>
<td>22.06 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>147.10 ± 23.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>288.32 ± 8.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.46 ± 1.59&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Pancreatin treatments</strong></td>
<td></td>
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<tr>
<td>10 min</td>
<td>9.43 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1294.70 ± 40.02&lt;sup&gt;e&lt;/sup&gt;</td>
<td>280.05 ± 15.16&lt;sup&gt;e&lt;/sup&gt;</td>
<td>62.20 ± 10.15&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 min</td>
<td>10.33 ± 0.18&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1577.90 ± 23.19&lt;sup&gt;e&lt;/sup&gt;</td>
<td>274.07 ± 17.63&lt;sup&gt;e&lt;/sup&gt;</td>
<td>61.97 ± 9.59&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 h</td>
<td>10.57 ± 0.38&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1813.90 ± 64.91&lt;sup&gt;e&lt;/sup&gt;</td>
<td>269.60 ± 8.56&lt;sup&gt;e&lt;/sup&gt;</td>
<td>49.48 ± 7.50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 h</td>
<td>12.07 ± 0.46&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1911.85 ± 139.80&lt;sup&gt;e&lt;/sup&gt;</td>
<td>264.96 ± 14.28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>41.39 ± 6.19&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h</td>
<td>20.91 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>463.32 ± 23.48&lt;sup&gt;e&lt;/sup&gt;</td>
<td>89.97 ± 10.11&lt;sup&gt;e&lt;/sup&gt;</td>
<td>16.61 ± 1.28&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Trypsin treatments</strong></td>
<td></td>
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<tr>
<td>10 min</td>
<td>14.86 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>553.07 ± 18.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>304.61 ± 14.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.93 ± 5.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 min</td>
<td>16.75 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>546.31 ± 30.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>308.14 ± 16.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.66 ± 6.09&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 h</td>
<td>16.47 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>506.10 ± 65.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>311.06 ± 6.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.91 ± 8.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 h</td>
<td>19.37 ± 0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>391.82 ± 32.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>339.00 ± 22.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.00 ± 11.52&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h</td>
<td>20.68 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>351.59 ± 29.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>452.62 ± 18.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.77 ± 10.44&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: DH, degree of hydrolysis; S₀, surface hydrophobicity; EAI, emulsifying activity index; ESI, emulsion stability index. Different lower case superscript letters in the same column represent significant differences (P<0.05) in the mean within the same enzyme treatment; the uppercase superscript letter A represents the mean is significantly different (P<0.05) from that of control sample, None (NaCas). Data values are expressed as mean ± standard deviation from three replicates.

Generally speaking, the buried hydrophobic amino acids become exposed during initial proteolysis, resulting in unfolded structures and increased S₀ of the hydrolysates, followed by the decrease of S₀ due to the disruption of these hydrophobic patches and resultant smaller peptide fragments. Among the three enzymes tested in the present study, only the pancreatin-treated hydrolysates exhibited this trend, while the S₀ values of papain- and trypsin-treated hydrolysates gradually decreased from the beginning of the hydrolysis process. This phenomenon may be explained by the higher enzymatic activity of papain and trypsin (Table 1), both of which are more effective at hydrolysing NaCas and thus resulted in hydrolysates with smaller molecular weights, as observed in SDS–PAGE (Fig. 1).
generally improved for most hydrolysate samples, with exceptions of ESI for hydrolysates produced by papain for more than 1 h and EAI of the hydrolysate produced by pancreatin for 24 h. EAI indicates how quickly a surfactant adsorbs onto the newly formed oil droplet surfaces, and smaller surfactants therefore typically have better emulsifying properties (Walstra, 2002). The proteolysis of NaCas resulted in a smaller casein mass and therefore an increased EAI (Table 1). The only exception was observed for the 24 h treatment by pancreatin, possibly due to the hydrolysates having little surface activity. Conversely, the EAI as measured showed the impacts of enzyme type and hydrolysis time, as expected based on the differences in primary structures (Fig. 1), and the overall correlation between ESI and $S_0$ was not observed.

To understand the physical bases of emulsion stability, the surface load (the amount of surfactant adsorbed on unit surface area of droplets), viscoelasticity of interfacial layer, solubility characteris-

**Fig. 1.** SDS–PAGE of sodium caseinate (lane 0) and its hydrolysates produced by papain, pancreatin or trypsin. In each group, lanes 1, 2, 3, 4, and 5 represent samples produced by hydrolysis for 10, 30 min, 1, 4, and 24 h, respectively. Lane 6 shows protein markers.

**Fig. 2.** The pH-dependent solubility of sodium caseinate before (A) and after hydrolysis by papain (B), pancreatin (C), or trypsin (D) for durations from 10 min to 24 h. Error bars are standard deviations from three replicates.
tics of the oil, surface charge of oil droplets and dimension and
distribution of oil droplets during extended storage period
(McClements, 2006) are to be characterized in the future.

3.5. Antioxidant properties

3.5.1. DPPH radical scavenging activity

DPPH is a relatively stable free radical that has been widely
used to evaluate antioxidant properties of proteins and their
hydrolydates (Klompong, Benjakul, Kantachote, & Shahidi, 2007).
Although relatively high DPPH scavenging activity was observed
in the first 30 min with salmon (Ahn, Je, & Cho, 2012) and trevally
fish (Klompong et al., 2007) proteins and their hydrolysates, NaCas
and hydrolysates had limited activity within 30 min, and were
therefore tested for up to 24 h (Fig. 3). The higher DPPH scavenging
activity was observed at a longer incubation time. The results sug-
gest caseins and their hydrolysates have limited electron-donating
capacity. Most hydrolysates showed higher DPPH scavenging
activity than NaCas. Overall, the DPPH scavenging activity of pa-
pain treatments was independent on the hydrolysis time and
was higher than the other two groups. Conversely, the DPPH
scavenging activity of pancreatin- and trypsin-treatments was
dependent on the hydrolysis time and was higher than that of
NaCas only after hydrolysis for a sufficiently long time (>1 h for
pancreatin and >4 h for trypsin treatments). The different scaveng-
ing activity of papain hydrolysates may be a result of its higher
enzymatic activity, but less specificity in hydrolysing NaCas, pro-
ducing low MW peptides without defined amino acid sequences.

3.5.2. Reducing power

The reducing power was evaluated to determine the ability of a
sample to donate electrons or protons, which has been reported to
be directly correlated with peptide cleavages (Cumby, Zhong,
Naczk, & Shahidi, 2008) and therefore the improved antioxidant
activities of hydrolysates. In the present study, the reducing power
was expressed as the ability to reduce Fe^{3+}/ferricyanide complex to
the Fe^{2+} form, which can be monitored for the absorbance of Perl’s
Prussian blue at 700 nm. As shown in Fig. 4A, the reducing power
of NaCas greatly improved under all enzymatic hydrolysis condi-
tions studied, generally more significant at longer hydrolysis time.
For the hydrolysates produced by 24 h hydrolysis, the pancreatin
treatment exhibited the greatest reducing power, which was
two-fold that of NaCas. The enhanced reducing power of hydroly-
sates may be related to the increased availability of protons and
electrons resulting from enzymatic hydrolysis.

3.5.3. Ferrous ion chelating activity

Metal chelating activity is another important functionality that
directly contributes to antioxidant properties of bioactive
compounds, because transition metals play a critical role in the
generation of free radicals, which can subsequently initiate autox-
idation. Among metal ions, ferrous ions have been associated with
oxidative damage in cells resulting in lipid peroxidation (Huang
et al., 2002). In the present study, the disintegration of ferrozine/
Fe^{2+} complex and thus the reduction of a violet colour was used
as an indicator to evaluate the activity of hydrolysates chelating
Fe^{2+} (Wu et al., 2003).

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Fig. 3. DPPH radical scavenging activity of sodium caseinate (A) and its hydrolysates during incubation with the DPPH solution at room temperature for 0.5–24 h (X-axis). The hydrolysates were produced by papain (B), pancreatin (C), or trypsin (D) for durations from 10 min to 24 h, as labeled in the legend. Error bars are standard deviations from three replicates. Asterisks above bars indicate the significant difference (\(P<0.05\)) from the sodium caseinate at the respective time point.
other studies based on porcine plasma protein (Liu, Kong, Xiong, & Xia, 2010) and trevally fish protein (Klompong et al., 2007). This discrepancy may be attributed to the specificity of proteases and the length and amino acid sequence of peptides.

3.5.4. Inhibitory activity on linoleic acid autoxidation

Linoleic acid autoxidation is a free radical-mediated oxidation via abstraction of hydrogen atoms from methylene carbons and has been extensively studied as a model for lipid peroxidation (Niki, 2009). In this study, the inhibitory activity on lipid peroxidation by hydrolysates was evaluated using the ferric thiocyanate method, and the estimated induction period (tind) of linoleic acid autoxidation was compared in Fig. 4C. The tind of the control was only 1 d, while NaCas and the hydrolysates exhibited much longer tind. The tind increased initially, from 5 d for NaCas to up to 9 d for the 30 min hydrolysis treatments, before decreasing to a value identical to NaCas after 24 h hydrolysis. For hydrolysates produced after 10–60 min hydrolysis, the pancreatin treatments were the most effective in prolonging the tind, while the other two groups exhibited similar tind. For hydrolysates produced after 4 and 24 h proteolysis, the enzyme type did not show any impact. The inhibitory activity against linoleic acid autoxidation was previously correlated to hydrophobic amino acids in the hydrolysates, which have higher affinity to the lipid phase than hydrophilic amino acids, and consequently can scavenge lipid-derived radicals to a greater extent (Pownall, Udenigwe, & Aluko, 2010). This is consistent with findings from the present study: the longer tind of the pancreatin treatments agreed with the higher S0 of the hydrolysates, with exception of the 4 and 24 h treatments, where the impact of low MW may outweigh the S0.

3.6. ACE-inhibitory activity

ACE has been recognised as a major factor leading to hypertension, due to its capability to hydrolyse the inactive decapptide angiotensin I to angiotensin II, a potent vasoconstrictor (López-Fandiño et al., 2006). Compounds inhibiting ACE thus have anti-hypertension potential, as reported for food protein-derived peptides. Long term consumption of milk products has been linked to the prevention of hypertension development, likely due to ACE-inhibitory peptides hydrolysed during the digestion of dairy proteins in vivo (López-Fandiño et al., 2006). The ACE-inhibitory activity of dairy protein hydrolysates is highly dependent on the protease used, because of enzyme specificity (Korhonen, 2009; Otte, Shalaby, Zakora, Prip, & El-Shabrawy, 2007).

The ACE-inhibitory activity of hydrolysates produced in the current study is shown in Fig. 5. The ACE-inhibitory activity of NaCas was significantly increased after proteolysis, by up to 9 times for the long time hydrolysis by papain and trypsin. The ACE-inhibitory activities of papain- and pancreatin-treated hydrolysates increased gradually with the increase of hydrolysis time, while the trypsin-treated ones reached a plateau after 1 h of hydrolysis. Pancreatin treatments had the lowest ACE-inhibitory activities, probably due to the lower proteolytic activity of pancreatin, as evidenced by relatively higher MW of hydrolysates. Findings from the present study agree with a previous report on the pepsin-treated casein hydrolysates (Miguel, Contreras, Recio, & Aleixandre, 2009), which further suggests that both in vitro and in vivo ACE-inhibitory activities are mainly contributed by the lower MW fraction of hydrolysates. Several bioactive peptides with potent ACE-inhibitory activity have been identified from casein hydrolysates in recent literatures. For instance, a tripeptide (Ile–Pro–Pro) and an octapeptide (Gln–Asp–Lys–Thr–Glu–Ile–Pro–Thr) have been isolated and identified from casein fractions hydrolysed by the protease from Bacillus subtilis (Welderufael, Gibson, & Jauregi, 2012). Another study pointed out that the peptides from α-casein fractions

![Fig. 4. Antioxidant activity of sodium caseinate (NaCas) before and after enzymatic hydrolysis for different durations, evaluated for (A) reducing power, (B) ferrous chelating activity, and (C) inhibitory effects against the induction period of linoleic acid autoxidation. In each antioxidant assay, asterisks above bars indicate significant difference (P<0.05) from NaCas. Error bars are standard deviations from three replicates. In panel C, the three replicates of each sample exhibited the identical induction period (day), and standard deviations are not given.](image-url)
4. Conclusions

Under the studied conditions, casein hydrolysates produced by three enzymes had different primary structures based on SDS–PAGE. The differences in hydrolysis kinetics and possibly specificity caused significant differences in physical, chemical and biochemical properties of hydrolysates among enzyme treatment groups. Proteolysis greatly improved the solubility of NaCas, eventually leading to a weak pH-dependence after extensive hydrolysis. The emulsifying property was generally improved after hydrolysis. The emulsion stabilizing property of hydrolysates showed a dependence on hydrolysis conditions, but the exact mechanisms are to be investigated. Antioxidant properties measured using DPPH radical scavenging, reducing power, and inhibition against linoleic acid autoxidation were improved after hydrolysis of NaCas, while the ability of chelating ferrous ions reduced. The ACE-inhibitory activities were also dramatically improved, likely resulting from lower MW fractions in hydrolysates. Although it is apparent that the primary structure of hydrolysates was highly dependent on the studied hydrolysis conditions, future work is needed to identify bioactive peptides responsible for the improved biochemical properties.

References


