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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.

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 αs1-, αs2-, β- and κ-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, Otte, & 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,

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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 BAEE units/mg protein), ACE (from rabbit lung), substrate peptide (hippuryl-histidyl-leucine, HHL), and 8-anilinonaphthalene-1-sulphonic 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-phthaldialdehyde (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:

$$DH\% = \frac{A_{sample} - A_{blank}}{A_{total} - A_{blank}} \times 100 \quad (1)$$

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 (S_0)

The S_0 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 $R^2 > 0.995$ was used as an index for S_0.

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 I.Q.^{2} microprocessor homogenizer