JAP&T
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Journal of Agroalimentary Processes and Technologies 2011, *17*(3), 308-314

Journal of Agroalimentary Processes and Technologies

Functional properties of sodium caseinate hydrolysates as affected by the extent of chymotrypsinolysis

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Received: 28 May 2011; Accepted: 28 July 2011

Abstract

Casein is the main proteinaceous component of milk, where it accounts for ca. 80% of the total protein inventory. Biologically active peptides can be produced from precursor milk proteins. The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole proteins.

The objectives of present study were to study the effect of enzymatic hydrolysis on the functional properties of sodium caseinate. Gastrointestinal enzymes, such as chymotrypsin, have been utilized to generate hydrolysates. Functional properties were limited to solubility, foaming capacity, and 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity. These properties were evaluated and compared as a function of hydrolysis degree.

Keywords: casein, enzymatic hydrolysis, functional properties

1. Introduction

A major role of milk proteins is to supply amine acids and nitrogen to the young mammal. Milk proteins also constitute an important part of dietary proteins for the adult. However, these proteins should not only be considered as nutrients for they probably have a physiological role as well.

Casein is the main proteinaceous component of milk, where it accounts for ca. 80% of the total protein inventory. Until recently, the main physiological role of casein in the milk system was widely accepted to be a source of amino acids required by growth of the neonate. However, the dominant physiological feature of the casein micelle system has more recently been proven to be the prevention of pathological calcification of the mammary gland [11]. Caseins can be an important source of biologically active peptides [15].

While no specific physiological property has been proposed for the whole casein system (or its individual fractions, for that matter), various peptides hidden (or inactive) in the amino-acid sequence have been the subject of increasingly intense studies. The peptides are not active within the parent protein but can be released and activated following enzymatic hydrolysis [6, 8].

Much work regarding those peptides, which are known to possess bioactivities, is currently underway regarding their release via selective enzymatic hydrolysis.

The beneficial health effects may be attributed to numerous known peptide sequences exhibiting antimicrobial, antioxidative, antithrombotic, antihypertensive, immunomodulatory and opioid activities, among others [6].

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The activity of these peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from 2 to 20 amino acid residues, and many peptides are known to reveal multi-functional properties. For above reasons, milk-derived bioactive peptides are considered as prominent candidates for various health-promoting functional foods targeted at heart, bone and digestive system health as well as improving immune defense, mood and stress control. Recent studies suggest that bioactive milk peptides may also be beneficial in reducing the risk of obesity and development of type two diabetes [31].

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. A large number of studies have demonstrated that hydrolysis of milk proteins by digestive enzymes can produce biologically active peptides [16]. The most prominent enzymes are pepsin, trypsin and chymotrypsin that have been shown to release a number of antihypertensive peptides, calcium-binding phosphopeptides (CPP), antibacterial, immunomodulatory and opioid peptides both from different casein (α-, β- and κ-casein) and whey proteins, e.g., α-lactalbumin, β-lactoglobulin and glycomacropeptide [7,9,10,19].

Proteins are used in all kinds of food products to profit from their nutritional value or from specific functional properties. Their physicochemical behaviour might, however, also impede the use of proteins in products, for example, in high-energy drinks, where high viscosity or limited solubility restricts the protein concentration. Protein functionality can be modified by enzymatic hydrolysis, which alters, for instance, solubility, viscosity, and emulsion and foam properties. Choice of enzyme and process conditions influence hydrolysate composition and thereby the functional properties.

The objectives of present study were to study the effect of enzymatic hydrolysis on the functional properties of sodium caseinate. The gastrointestinal enzyme chymotrypsin has been utilized to generate hydrolysates. Functional properties were limited to solubility, foaming capacity, and 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity. These properties were evaluated and compared as a function of hydrolysis degree.

2. Materials and Methods

Casein sodium salt from bovine milk and α-chymotrypsin (EC 3.4.21.1, type II from bovine pancreas, 40 units/mg protein) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All solvents and chemical reagents were of analytical grade.

*Enzymatic hydrolysis.*A 5% (w/v) aqueous solution of casein sodium salt was adjusted to pH 8.0 with HCl 1 mol.L^{-1} and digested with 0.25% (w/w of substrate) α-chymotrypsin for 10 to 80 min at 37°C. The reaction was terminated by heating at 70°C for 15 min and the pH was adjusted to 4.6 by addition of HCl 1 mol.L^{-1}. The digest was centrifuged at 6000xg for 20 min and the supernatant was freeze dried until use.

*Hydrolysis degree.*The DH% values were determined by the modified *o*-phtaldialdehyde (OPA) method. This involved using N-acetyl cysteine (NAC) as the thiol reagent [25]. The OPA/NAC reagent (100 mL) was prepared daily by combining 10 mL of 50 mm OPA (in methanol) and 10 mL of 50 mm NAC, 5 mL of 20% (w/v) sodium dodecyl sulphate (SDS), and 75 mL of borate buffer (0.1 M, *p*H 9.5). The reagent was covered with aluminum foil to protect from light and allowed to stir for at least 1 h before use.

The OPA assay was carried out by the addition of 10 μL of sample during hydrolysis to 2.5 mL of OPA/NAC reagent. The absorbance of the solution was measured at 340 nm with an UV–VIS GBC Cintra 202 spectrophotometer. The absorbance values for the interaction of amino groups with OPA were taken after 2 min standing for un-hydrolyzed sample and after 10 min standing for hydrolyzed samples. A standard curve was prepared using L-leucine (0-1.5 mM).

The DH value was calculated based on equation 1:

$$\text{DH}\ (\%) = \frac{\Delta A \times M \times d}{\varepsilon \times c} \times \frac{100}{N} \qquad (1)$$

where Δ Abs is the Abs of test sample at 340nm - Abs un-hydrolyzed sample at 340 nm, M the molecular mass of the test protein (Da), d the dilution factor, e the molar extinction coefficient at 340 nm (6000 L mol^{-1} cm^{-1}), c the protein concentration (g.L^{1}) and N the total number of peptide bonds per protein molecule.

*2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity.*DPPH radical-scavenging activity was measured, using the method of Yen and Wu [29]. Sodium casein hydrolysates with different DHs were dissolved in distilled water to obtain a concentration of 40 mg protein/ml. To 4 ml of sample solutions, 1.0 ml of 0.2 mM DPPH was added and mixed vigorously. After incubating for 30 min, the absorbance of the resulting solutions was measured at 517 nm using a spectrophotometer (UV-VIS Cintra 202, GBC Scientific Equipment, Australia).

The control was conducted in the same manner, except that distilled water was used instead of sample. DPPH radical scavenging activity was calculated according to the equation 2 [29,30]:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{(1-(A_{517}\ sample - A_{517} control))}{A_{517}\ control} \times 100 \quad (2)$$

*Solubility.*To determine protein solubility 1 g of protein hydrolysate sample was dispersed in 100 ml of distillated water and pH of the mixture was adjusted to 2, 4, 6 and 8 with HCl 1 N and NaOH 1 N. The mixture was stirred at room temperature for 15 min and centrifuged at 6000xg for 15 min. Protein contents in the supernatant were determined using the Lowry method. Protein solubility was calculated according to the equation 3:

$$\text{Solubility (\%)} = \frac{SP}{TP} x100 \quad (3)$$

where SP is the protein content in supernatant and TP is the total protein content in sample.

*Foaming properties.*Foaming capacity and stability of protein hydrolysates were determined according to the method of Sathe and Salunkhe [23]. One hundred milliliters of 1% sample solution were whipped to incorporate the air for 3 min at room temperature. The whipped sample was immediately transferred into a 500 ml cylinder and the total volume was read after 30 s. The foaming capacity was calculated according to the equation 4:

$$\text{Foaming capacity (\%)} = \frac{A-B}{B} x100 \quad (4)$$

where A is the volume after whipping (ml) and B is the volume before whipping (ml).

The whipped sample was allowed to stand at room temperature for 3 min and the volume of whipped sample was then recorded. Foam stability was calculated according to the equation 5:

$$\text{Foam stability (\%)} = = \frac{A-B}{B} x100 \quad (5)$$

where A is the volume after standing (ml) and B is the volume before standing (ml).

3. Results and discussions

*Hydrolysis degree.*Hydrolysis of casein sodium salt using α-chymotrypsin was carried out by the OPA/NAC method. Rapid hydrolysis was observed within the first 30 min of reaction. Thereafter, a slower rate of hydrolysis was recorded. The maximum extent of hydrolysis (DH 8.7±0.7%) was observed after 60 min of hydrolysis (Figure 1). It seems that casein sodium salt is relative resistance to proteolysis, which is possible due to the compact tertiary structure of the protein that protects most of the enzyme susceptible peptide bonds.

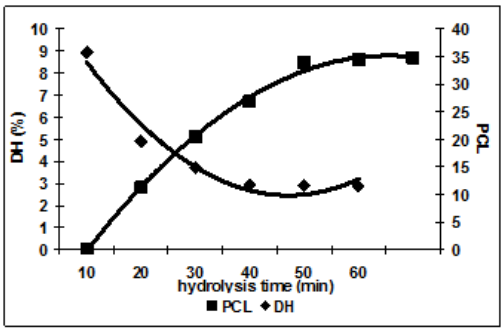

Figure 1. Hydrolysis curve of casein sodium salt by α-chymotrypsin

From the degree of hydrolysis the average peptide chain length (PCL) of hydrolysates can be estimated according to equation 6, assuming that the entire hydrolysate s are soluble [1].

$$\text{PCL} = \frac{100}{DH\%} \quad (6)$$

The peptide chain length is related to the average molecular weight of peptides in hydrolysates. However, hydrolysates with similar PCL may have substantially different peptide molecular weight distributions. Enzyme specificity, for example, influences the peptide size distribution: a pure endoprotease will yield peptides with varying lengths, whereas an enzyme mixture that is mainly composed of exopeptidases will yield hydrolysates with mostly free amino acids combined with remaining large peptides.

Moreover, proteases acting according to a one-by-one mechanism will yield hydrolysates with other peptide length distributions than proteases that hydrolyse proteins according to a zipper type reaction.

Given the relative higher resistance to proteolysis of casein sodium salt, it was decided to test the influence of thermal treatment on the protein susceptibility to enzymatic hydrolysis at neutral pH. Thus, protein solutions were subjected to heat treatment in the temperature range of 85 to 95°C for 15 minutes, followed by cooling in ice water (2 min) and hydrolysis at 37°C for one hour. In these experiments, a higher enzyme substrate ratio (1:50) was used. The results are given in Figure 2. It can be seen a significant increase in the DH after heat pretreatment at 85°C and 90°C. For example, DH was about four times greater after 60 minutes of hydrolysis in the case of thermal pretreatment of the protein solution at 90°C.

At 95°C, the increase in DH was significant in the first 10 minutes of hydrolysis, followed by a sequential decrease in the rate of hydrolysis. Even in these cases, DH values were higher when compared with the un-treated solutions.

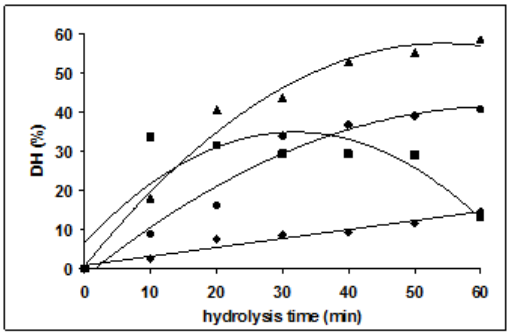

Figure 2. The heat-induced changes in DH (◆ no thermal treatment, ● 85°C, ▲ 90°C, ■ 95°C)

*DPPH radical-scavenging activity.*DPPH radical-scavenging activities of hydrolysates with different DHs are depicted in Figure 3.

Bars represent standard deviations from duplicate determinations. It can be seen that hydrolysates with DH between 5 and 8.7% exhibited the highest DPPH radical-scavenging activity. As the hydrolysis time increased to 80 min, DPPH radical-scavenging activity decreased. Nevertheless, no significant differences were observed for the first 60 min of hydrolysis.

Antioxidative activity of protein hydrolysates depends on the proteases [14] and hydrolysis conditions employed [20].

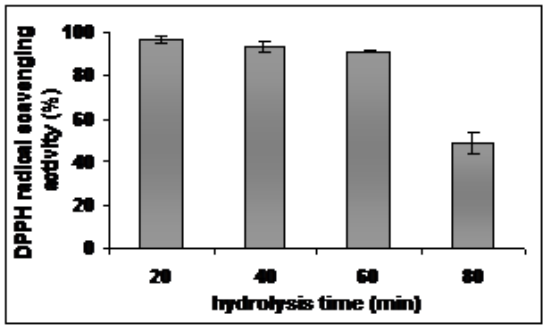

Figure 3. DPPH radical-scavenging activity of sodium casein hydrolysates as a function of hydrolysis time

Wu et al. [28] suggested that during hydrolysis, a wide variety of smaller peptides and free amino acids is generated, depending on enzyme specificity. It seems that changes in size, level and composition of free amino acids and small peptides affect the antioxidative activity.

Research on various casein hydrolysates has shown that they possess radical scavenging activities [4,5]. Suetsuna et al. [25,26] found that casein hydrolysates exerted scavenging activity towards the superoxide anion, hydroxyl radical, and DPPH radical. It is suggested that the casein-derived Glu-Leu sequence is important for this radical scavenging action, thus the primary structure of the protein plays a role in determining the antioxidant activity.

Although Jing and Kitts [13] showed that casein does not affect cellular enzymes, Laparra et al. [17] reported that CPP reduced glutathione (GSH) concentration and increased GSH-reductase activity in Caco-2 cells. In addition, Phelan et al. [21] reported that casein hydrolysates affected both cellular catalase activity and GSH content in Jurkat cells. They found that casein hydrolysates contained a certain degree of electron donating capacity as determined by the ferric reducing antioxidant power (FRAP) assay (17–32 mmol L^{-1}) [22].

*Solubility.*The solubilities of sodium casein hydrolysates with different DH in the pH range of 2 to 8 are given in Figure 4. All hydrolysates were highly soluble over a wide pH range with more than 90% solubility.

In general, the degradation of proteins to smaller peptides leads to more soluble products.

Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates. As it can be seen from Figure 2, the hydrolysates solubility varies slightly as a function of DH and pH. It should be mentioned that the solubility values were quite high at pH 4.

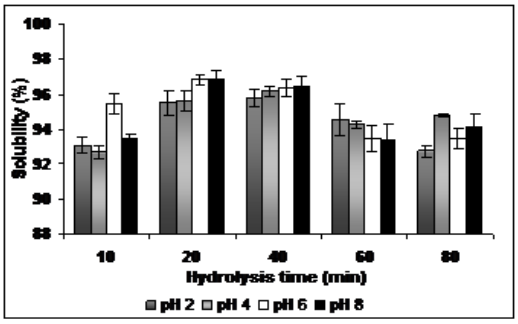

Figure 4. Solubility of protein hydrolysates as influenced by pH values
(Bars represent standard deviations from two repetitions)

The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points. Solubility variations could be attributed to both net charge of peptides that increase as pH moves away from pI and surface hydrophobicity that promotes the aggregation via hydrophobic interaction. Due to the high solubility of the sodium casein hydrolysates over a wide pH range, it was presumed that products had a low molecular weight and were hydrophilic in nature.

*Foaming properties.*Enzymatic hydrolysis of sodium caseinate caused an increase in the foam capacity initially and then a decrease with hydrolysis time. The foam volume was found to be similar at both control and 10 min of hydrolysis, with a slightly decrease after 40 min (Figure 5).

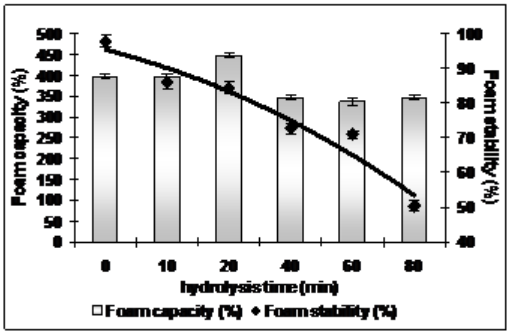

Figure 5. Foaming capacity and foam stability of sodium casein hydrolysates
(Bars represent standard deviations from two repetitions).

The foam stability of the control was greater than that of the treated samples. Samples treated with α-chymotrypsin showed gradual decrease in foam stability with an increase in chymotrypsinolysis time. The lowest value of foam stability was measured after 80 min of hydrolysis.

Hydrolysis of casein generally resulted in increased foam-forming ability of the hydrolysates compared to the parental proteins [2,3,18]. The effect of hydrolysis on foam stability seems to depend on enzyme specificity and degree of hydrolysis [18,24].

The above results show that a limited amount of hydrolysis is desirable to increase foaming but foam stability is greatly decreased as a result of such hydrolysis. This is probably due to an initial increase in the polypeptide content, which allows more air to be incorporated. However, the polypeptides do not have the strength required to give stable foam. The decrease in foam stability manifests itself primarily in the initial 60 min of reaction. Further hydrolysis is likely to result in peptides, which lack the ability to stabilize the air cells of the foam.

Hydrolysis of casein results in a reduction of molecular weight, which might promote foam formation due to the faster diffusion of molecules to the interface [27]. On the other hand, peptides formed during hydrolysis might destabilize protein foams by displacement of proteins or by disturbing protein/protein interactions [27, 30]. Furthermore, hydrolysis leads to increased charge density, which negatively influence foam stability, because foam stability was shown to improve when electrostatic repulsion of proteins is minimal [12].

4. Conclusions

Some functional properties of sodium caseinate hydrolysates were tested in this study. DPPH radical-scavenging activity of protein hydrolysates varied slightly with the DH. The antioxidant activity decreases an advanced hydrolysis. All hydrolysates were highly soluble over a wide pH range with more than 90% solubility. The foaming properties of the protein hydrolysates were a function of hydrolysis degree. Further studies are needed to identify peptides from hydrolysates and their complete characterization.

Acknowledgement

The authors acknowledge financial support from the National University Research Council (NURC, PN-II-ID-PCE-2008-2, Idea, ID 517) Romania.

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