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The effect of pH and thermal treatment on some functional properties of whey proteins hydrolysates as measured by fluorescence spectroscopy

Alina Hînţoiu*, Gabriela Râpeanu, Silvius Stanciu, Nicoleta Stănciuc

"Dunărea de Jos" University of Galaţi, Faculty of Food Science and Engineering, 800201, Domneasca Street, No. 111, Galati, Romania Romania

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Abstract

Milk protein represents an important source of amino acids and peptides in the human diet. In recent years, whey has gained immense recognition as a protein source in functional foods, infant formulas, and bakery products.

The objectives of this study were to investigate some functional properties of whey protein hydrolysates obtained with chymotrypsin as a function of pH and thermal treatment. The extent of hydrolysis and conformational changes induced by pH and temperature were monitored using intrinsic fluorescence intensity and anisotropy.

Keywords: pH, proteins hydrolysates, fluorescence spectroscopy

1. Introduction

Milk protein represents an important source of amino acids and peptides in the human diet. In recent years, whey has gained immense recognition as a protein source in functional foods, infant formulas, and bakery products [3]. Whey protein hydrolysates are extensively being prepared and used for nutritional support of human patients with various physiological insufficiencies and anomalies [7]. The main constituents are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), two small globular proteins that account for approximately 70–80% of total whey protein. Native whey proteins are not fully hydrolyzed by digestive enzymes because of the presence of disulfide bonds and other chemical barriers in their structures [19], which possess antigenic activities for humans [9]. In particular, *β*-LG can induce a milk antigenic response in human infants because of their underdeveloped gastrointestinal tracts [22] and immune systems [16].

Whey protein concentrates with ~80% protein are frequently used to fortify various food products, owing to their low fat and lactose contents and high protein levels.Enzymatic hydrolysis of whey protein offers a practical way to reduce its antigenicity [8]. Furthermore, it can yield a variety of new peptides that may offer many physiological benefits for humans [12]. These hydrolysates can be added to special foods to increase value, as enzymatic hydrolysis can optimize their functional properties (e.g., gelling, emulsifying and foaming capacities and solubility) [4,23]. Thus, there is considerable commercial interest in the preparation of whey proteins for food, nutraceutical, and therapeutic applications [19].

When developed as food ingredients, the processing of these peptides is vital to their activity. For example, heat treatments will have negative effects on the bioavailability of whey peptides, so processors must carefully monitor production parameters.

Corresponding author: *e-mail:* : Nicoleta.Sava@ugal.ro

Appropriate selection of enzymes for proteolysis will result in maximum biological activity and will limit the development of bitter flavour notes, with low cost. Digestive enzymes are an interesting for the liberation of bioactive peptides, and also for the improvement of protein digestibility and for decreasing protein allergenicity, due to its specificity [15]. Additionally, it is important to study the functional properties of hydrolysates, because hydrolysis produced with different proteolytic enzymes can increase solubility and change gelling properties.

Some enzymes can induce gelation following whey protein hydrolysis; others impair gelling properties [5,6,13]. Gels confer structure, texture and stability to food products; they also allow the retention of large quantities of water and other small molecules inside the food matrix. These aspects are appreciated by processed food manufacturers.The objectives of this study were to investigate some conformational changes of whey protein hydrolysates obtained with chymotrypsin as a function of pH and thermal treatment. The extent of hydrolysis and conformational changes induced by pH and temperature were monitored using intrinsic fluorescence intensity and anisotropy.

2. Materials and Methods

Whey protein concentrate (WPC; 93% w/w protein, 80% β-LG) was obtained from Kuk Romania. 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 WPC was adjusted to pH 7.5 with HCl 1 mol.L^{-1} and digested with 0.25% (w/w of substrate) α-chymotrypsin for 10 to 30 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.

*Thermal treatment.*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. 5 ml of hydrolysates solutions were filled in glass tubes (1 cm diameter). The thermal treatment experiments were conducted in a thermostatic water bath at 70 and 85^{0}C for 15 min. After thermal treatment, the tubes were immediately cooled in ice water to prevent further denaturation and centrifuged at 6000xg for 15 min. Protein contents in the supernatant were determined using the Lowry method.

*Intrinsic fluorescence spectroscopy.*Intrinsic fluorescence measurements in supernatants were made on a LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT) using an excitation wavelength of 292 nm and emission was collected between 300 and 420 nm. The excitation and emission slits were both 10 nm, and the scan speed was 200 nm min^{-1}. Measurements were made at 25 °C.

*Fluorescence Anisotropy measurements.*For the fluorescence anisotropy experiments, the wavelengths of excitation and emission were set at 292 nm with a slit of 10 nm and 340 nm with a slit of 10 nm, respectively. The fluorescence anisotropy is defined as:

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} - 2GI_{VH}}$$

where I_{VV} is the fluorescence intensity recorded with excitation and emission polarization in vertical position, and I_{VH} is the fluorescence intensity recorded with the emission polarization aligned in horizontal position. The G factor is the ratio of sensitivities of detection system for vertically and horizontally polarized light.

3. Results and discussion

Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis.

In proteins, Phe, Tyr and Trp absorb UV radiation up to an excitation. Restoring native state can occur through:

a) fluorescent or phosphorescent emission;
b) energy transfer to another center absorption, for example from Tyr to Trp.

Since it was revealed that the major protein component (β-LG) can primarily determine the functional behavior of whey protein concentrate, and taking into account that the amount of β-LG in WPC is 80% (w/w), we considered that the hydrolysis pattern of WPC is governed primary by this protein.

Fluorescent studies allow measurement of changes in conformation of protein molecules, thereby influencing the emission wavelength (λ_{max}) and the fluorescence intensity at λ_{max} (I_{Trp}). In the monomer, the two Trp residues (19 and 61) are at a distance of 24 Å, while in dimer, Trp residues are located within a smaller distance of 21 Å. Theoretically, both the remaining Trp^{19} and Trp^{61} have the same capacity for absorption and emission of radiation. The two residues are found in two different environments. The remaining Trp^{19} is in a polar environment inside the protein molecule, while Trp^{61} is located at the surface rest of the molecule, very close to Cys^{66}-Cys^{160} disulphide bridge (distance between Trp^{19} and Cys^{160} is 3.9 Å) [1].

In the literature, there is controversy regarding the position of hydrophobic residues responsible for intrinsic fluorescence of β-LG. Manderson et al. [14] suggested that the remaining Trp^{19} is the main molecule responsible for intrinsic fluorescence of native β-LG. On the other hand, Iametti et al. [10] and Stapelfeldt et al., [21] observed that Trp^{61} is the most sensitive parameter for determining hydrophobicity of the exposed regions in protein structure. Iametti et al. [11] suggested that the dissociation of the dimer, regardless of which method is induced, involving a rearrangement of secondary structure. The distance between the groups change, but not the environment nature close to Trp residues in positions 19 and 61. Also, the remaining Trp^{61} secondary amine remains protonated at neutral pH. Crossing the Trp residues from a non-polar environment in a polar one, changes the excitation wavelength of β-LG solutions. Creamer [2] hypothesized that the dimer dissociation at pH 7.5 does not change the accessibility of Trp residues to fluorescent agent.The pH dependence of structural changes of WPC hydrolysates as a function of hydrolysis time was characterized in Figure 1 using fluorescence spectroscopy.

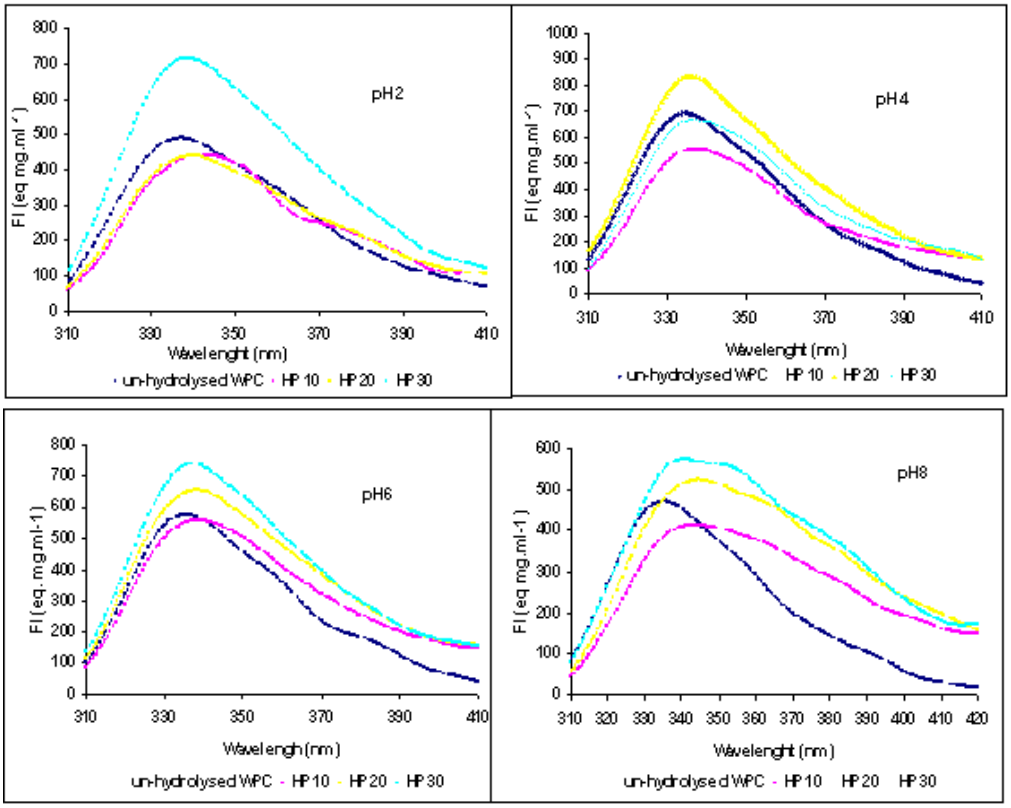

Figure 1. Fluorescence emission spectra of WPC hydrolysates as a function of pH and hydrolysis degree (◆ un-hydrolysed WPC, □ HP 10, □ HP 20, □ HP 30, □ HP 60)

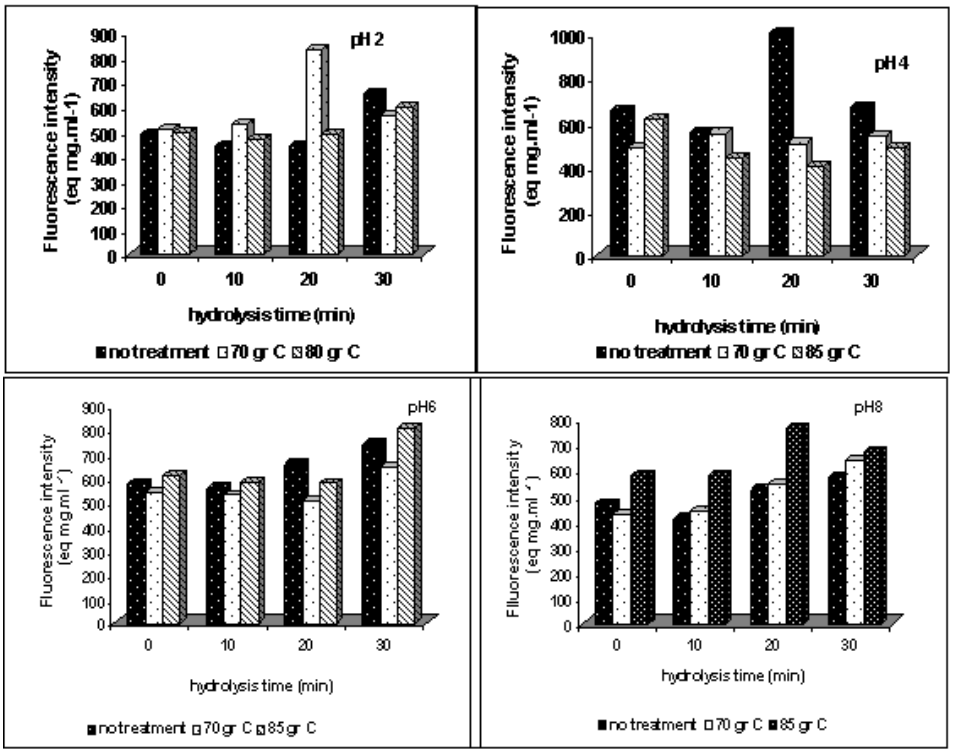

Figure 2. The maximum fluorescence intensity of the hydrolysates as a function of temperature

The conformational changes of whey protein hydrolysates induced by pH and heat treatment modified the maximum emission wavelength from 337 nm to 341 nm (pH 2), from 334.5 to 339 nm at pH 4, from 335 nm to 339.5 nm (pH 6) and from 335.5 nm to 352 nm at pH 8.

It should be noted that heat treatment at pH 8 resulted in a total exposure of Trp, λ_{max} value being with 2 nm higher than the maximum theoretical value obtained for the exposure of Trp in aqueous solution, in presence of urea (350 nm) [2]. This phenomenon has been recorded after 60 minutes of hydrolysis, and also for the hydrolysate obtained after 30 minutes of hydrolysis and heat-treated at 85^0C respectively. In fact, the dislocation of the maximum emission is accompanied by the displacement of the on-set of the emission band. As we know that Trp[19], located in the hydrophobic core of β-lactoglobulin, contributes to 75% of the total fluorescence emission, at the blue edge of the steady-state fluorescence spectra [17], we may consider that the dislocation of the on-set of the emission band is due to a higher exposure of Trp[19] to the aqueous solvent phase.

This behavior reflects the changes in proteins structure due to enzymatic hydrolysis. Simultaneously, a decrease in the fluorescence emission is observed after 10 minutes o hydrolysis, as a consequence of the increase in the dielectric constant of the environment (higher exposure to water) in the vicinity of the tryptophan(s).

*Fluorescence anisotropy.*Figure 3 presents the pH-dependences of the fluorescence anisotropy of WPC hydrolysates over the acidic and alkaline pH range, respectively.

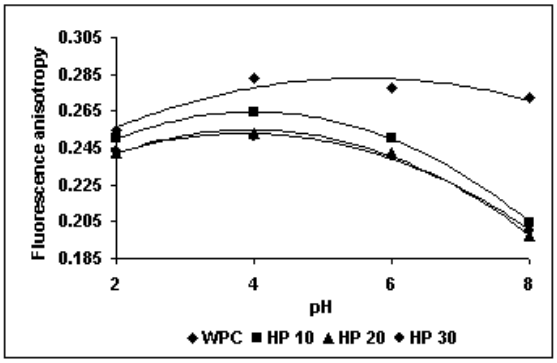

Figure 3. Fluorescence anisotropy of WPC hydrolysates as a function of pH

Anisotropy allows information to be acquired regarding the mobility of the fluorescence probes present in the peptides chains, which can be correlated to structural changes of hydrolysates induced by the pH and thermal treatment. Therefore, a decrease in fluorescence anisotropy is compatible with an increase in the average mobility of the tryptophans, suggesting that the peptides are present in a more unfolded state, whereas an increase in fluorescence anisotropy is indicative of a decrease in the average mobility of the tryptophans, resulting from structural packing or peptides aggregation.

Inspection of Figure 4 reveals that in the native state, WPC hydrolysate undergoes small temperature-induced transitions. The small decrease of fluorescence anisotropy may correspond to the unfolding of whey proteins. It seems that at pH 2, the aggregation of peptides molecules are favoured at 85^0C for the hydrolysates obtained after 20 minute of enzymatic reaction.

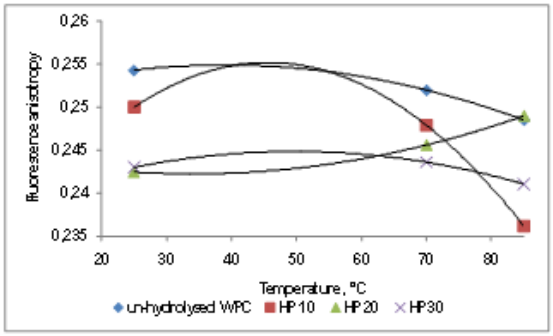

Figure 4. Fluorescence anisotropy of WPC hydrolysates at pH 2 as a function of temperature

In figure 5 are given the results for fluorescence anisotropy after heat treatment at pH 4. In the native state, the protein molecules are quite termostable given the small decrease of the fluorescence anisotropy values.

The aggregation seems to be favored for the large peptides obtained after 10 minutes of hydrolysis. Fluorescence anisotropy does not depend directly on the protein concentration, but it may be influenced by protein aggregation. Therefore, an indirect dependence on protein concentration may be expected. Actually, peptides aggregation phenomena are expected to be higher in the hydrolysates samples with a lower hydrolysis degree (HP 10).

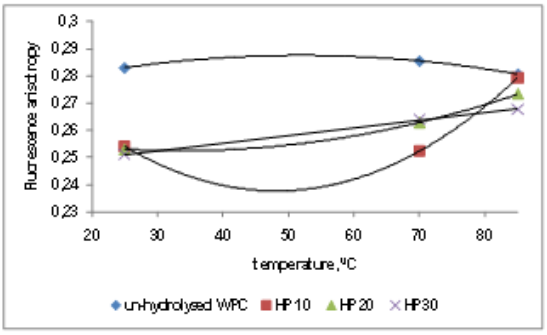

Figure 5. Fluorescence anisotropy of WPC hydrolysates at pH 4 as a function of temperature

The decrease in fluorescence anisotropy is, however, more accentuated at higher pH, indicating a significant increase in tryptophan mobility (figure 6). However, then heat treatment was performed at pH 6, the aggregation seems to be favored especially at higher temperature.

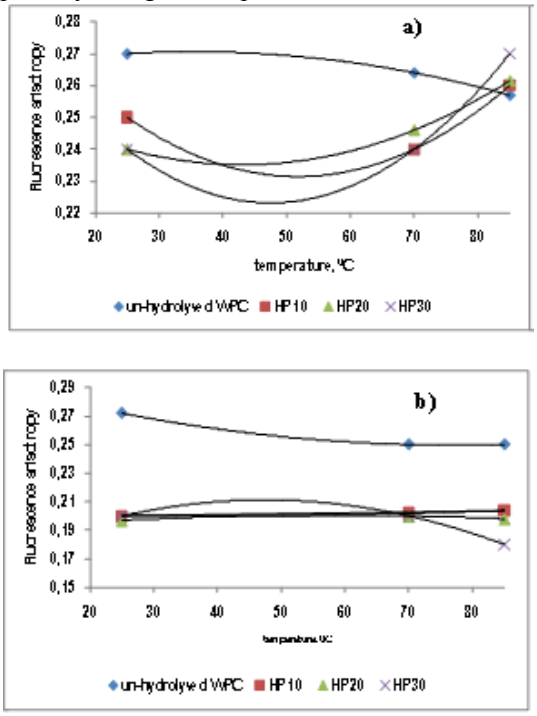

Figure 6. Fluorescence anisotropy of WPC hydrolysates at pH 6 (a) and 8 (b) as a function of temperature

The fluorescence anisotropy results agree well with those obtained by intrinsic fluorescence spectroscopy. In fact, the red shift of the fluorescence emission maximum observed at pH 8 clearly indicates the unfolding of the peptides and consequent exposure of Trp residues to the aqueous phase.

4. Conclusions

Our results demonstrate that intrinsic fluorescence technique and fluorescence anisotropy allow for the identification of different molecular processes involved during pH and temperature induced denaturation of proteins/peptides, as well as the identification of differences in the state of the protein heterogeneity resulting from denaturation.

Using these methods it was possible to identify variations of the structural heterogeneity (broadening of emission bands) of the solutions of whez proteins hydrolysates, while probing the changes of the environment in the vicinity of the tryptophans (red-shift of the emission band on-set and emission maximum). Identically, changes in the mobility of tryptophans constitute a direct and clear evidence of the changes incurred by whey proteins hydrolysates when submitted to the different denaturant conditions.

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