Innovative Romanian Food Biotechnology *Vol. 7, Issue of September, 2010*

Received May 10, 2010 / Accepted August 29, 2010

RESEARCH ARTICLE

THERMAL TREATMENT CAN MODIFY THE SUSCEPTIBILITY OF WHEY PROTEIN CONCENTRATE TO ENZYMATIC HYDROLYSIS

Nicoleta STANCIUC*, Alina HINTOIU, Silvius STANCIU and Gabriela RAPEANU

Department of Bioengineering, Faculty of Food Science and Engineering,

"Dunarea de Jos" University, Domneasca 111, 800201, Galati, România

Abstract

Susceptibility to enzymes hydrolysis is used as an index of flexibility since partial unfolding of protein molecules generally results in increased hydrolysis rate. Two set of experiments were performed on the effect of thermal treatment in the temperature range of 60-85°C on the susceptibility of whey protein concentrate to enzymatic hydrolysis by Proteinase K at 37°C and *p*H 7.5 and 8.0. Heat treatment in a certain time-temperature combination results in a significant increase in degree of hydrolysis, as monitored by OPA-NAC technique.

The time-dependent changes in the degree of hydrolysis were described by a fractional conversion model, which enables calculations of activation energy.

Whey proteins concentrate seems to be heat-sensitive for enzymatic hydrolysis, confirming that during heat treatment at neutral/alkaline *p*H the substrate is changing, since the dimmer dissociates with the exposure of hydrophobic residues.

Keywords: heat treatment, whey protein concentrate, enzymatic hydrolysis

Introduction

Whey proteins are widely used as food ingredients because of their good functional and nutritional properties (Ye and Taylor, 2009). Whey proteins confer a wide range of health benefits and are high in branched-chain amino acids, which increase satiety, protect against muscle-protein loss, enhance muscle-protein synthesis, and improve glycemic control (Ha and Zemel, 2003). The nutritional benefits of whey proteins appeal to a range of consumers from athletes to those concerned with having a healthy weight.

Some of the peptide fragments of milk proteins, which are normally inactive when buried within the sequence of native proteins, can be released in vivo during gastrointestinal digestion or in vitro through fermentation of milk or by the action of non-specific enzymes and may reveal anticancer, antihypertensive, antimicrobial and opioid

*Corresponding author: nicoleta.sava@ugal.ro

activities, and cholesterol lowering ability (Chobert, 2003).

Whey proteins, which consist principally of β-lactoglobulin, α-lactalbumin and bovine serum albumin, have globular structures. Upon heat treatment above 70^0C, these proteins unfold and aggregate (Sava *et al.,* 2005; Singh and Creamer, 1991).

β-lactoglobulin (β-LG) is the main protein in whey, constituting about 50% of the total whey proteins in bovine milk. Because of this structure, many authors consider β-LG responsible for the allergenic reaction because of its absence in human milk (Guo *et al*., 1994). Native protein is not hydrolyzed easily by pepsin, but is susceptible for chymotrypsin and trypsin hydrolysis, under non-denaturing conditions (Phillips *et al.,* 1994). The relative resistance to proteolysis is generally explained by the compact tertiary structure of the protein that protects most of the enzyme susceptible peptide bonds. Physical and/or chemical denaturation of β-LG generally leads to a higher rate of hydrolysis by various enzymes (Reddy *et al.,* 1988, Iung *et al.,* 1991; Dufour *et al.,* 1992; Chobert *et al.,* 1995; Stapelfeldt *et al.,* 1996; Maynard *et al.,* 1998; Kananen *et al.,* 2000; Sitohy *et al.,* 2001, Stanciuc *et al.,* 2008). After denaturation, the β-LG conforms may represent the ideal substrates for the proteases, because ample regions of the hydrophobic protein core are unfolded and accessible for the enzyme, contrarily to what happens in the native form or in the aggregated products of extensive thermal denaturation (Iametti *et al.,* 2002).

Proteinase K (EC 3.4.21.64), produced by the fungus *Tritirachium album Limber* is a serine protease that exhibits a very broad cleavage specificity. It cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids and is useful for general digestion of protein in biological samples.

The aim of this study was to follow the heat induced changes in the susceptibility of WPC to enzymatic hydrolysis at neutral pH for the heat-treated protein solutions. The degree of hydrolysis (further referred to as DH) obtained with Proteinase K was used as a parameter to quantitatively describe the effect of heating on the susceptibility of β-LG to proteolysis.

Materials and Methods

Whey protein concentrate (WPC; 93% w/w protein, 80% β-LG) was obtained from Kuk Romania. Proteinase K from *Tritirachium album Limber* was purchased from Sigma Aldrich. All solvents and chemical reagents were of analytical grade.

Heat treatments

Aqueous solutions of WPC were filled in the plastic tubes (1 cm diameter). The thermal treatment experiments were conducted in a thermostatic water bath.

Two sets of experiments were performed:

1) 0.5 ml of 2.5 mg/ml WPC, *p*H 7.5 were heat treated at various constant temperatures (60-85°C) for preset time intervals (0 – 30 minutes).

2) 1 ml of 25 mg/ml WPC, *p*H 8.0 were heat treated at various constant temperatures (60-80°C) for 15 minutes.

After thermal treatment, the tubes were immediately cooled in ice water to prevent further denaturation. The changes in susceptibility of WPC to enzymatic hydrolysis were measured exactly in 2 min after thermal treatment.

Enzymatic Hydrolysis of WPC solutions

The susceptibility of WPC to enzymatic hydrolysis was determined in aqueous dilutions after heat treatment, by adding 100 µl enzymes solution (1 mg/ml Proteinase K in 5 mM NaCl and 10 mM CaCl2, *p*H 8.0) to (un) heat-treated samples. The DH% values were determined by the modified o-phtaldialdehyde (OPA) method. This involved using N-acetyl cysteine (NAC) as the thiol reagent (Spellman *et al.,* 2003). The OPA/NAC reagent (100 mL) was prepared 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 to 2 mL of OPA/NAC reagent. The absorbance of this 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 WPC and after 10 min standing for hydrolyzed WPC. A standard curve was prepared using L-leucine (0-1.5 mM).

The DH value was calculated based on equation 1:

$$DH(\%) = \frac{\Delta Abs \cdot M \cdot d}{e \cdot c} \cdot \frac{100}{N} \quad \textbf{(1)}$$

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) and N the total number of peptide bonds per protein molecule.

The experiments were performed in duplicate. The standard deviation values were calculated using Microsoft Excel Office programme and were lower than 2%.

Kinetic data analysis

The fractional conversion model (a modified first order kinetic model) was used to define the heat-induced changes on the susceptibility of WPC to enzymatic hydrolysis at *p*H 7.5. In this model, the changes in DH after 10 minutes of hydrolysis (DH10) as a function of heating time is described by **equation 2** (Van der Plancken *et al.* 2003):

$$DH10_t = DH10_\infty + (DH10_\infty - DH10_i)\exp(-kt) \quad \textbf{(2)}$$

with $DH10_\infty$ is the equilibrium value for DH10 at infinite heating time and $DH10_i$ is the degree of hydrolysis of the samples at time 0 of thermal treatment.

The temperature dependence of the rate constant, k (min^{-1}) was described by the Arrhenius **equation (3)**:

$$k = k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \quad \textbf{(3)}$$

with T and T_{ref} the absolute temperature (K) and the reference temperature (K), respectively; k_{ref} the rate constant at T_{ref}, E_a the activation energy ($kJmol^{-1}$), R the universal gas constant (8.314 $Jmol^{-1}K^{-1}$). Kinetic parameters were estimated by nonlinear regression analysis (SAS Institute, 1999-2001).

Results and Discussion

Whey proteins are an excellent source of dietary nitrogen and essential amino acids. They also act as techno-functional ingredients in many formulated food systems due to their good solubility, surface activity, and gelling properties. In addition to their "classical" nutritional and techno-functional attributes, whey proteins and their associated peptides display significant functional food ingredient potential.

Thermal processing is the most common treatment applied in food industry, especially for the safety assurance of products. Heating may also improve the utilization of valuable food components, e.g. by improvement of susceptibility of the protein to enzymatic hydrolysis.

To investigate the effects of temperature on the subsequent susceptibility of WPC to proteolysis, in the first set of experiments samples were heated at various constant temperatures for 0-30 min.

Heat-treatments of WPC solutions between 65-80°C result in structural unfolding of protein conformation that gradually increased the subsequent extent of proteolysis by Proteinase K up to 75^0 C followed by a decrease in DH% at higher temperature (Figure 1).

Hydrolysis experiments were carried out at 7.5 and 37°C, by adding 100 µL of enzyme solution to 0.5 mL of 2.5 mg/ml WPC pre-heated solutions.

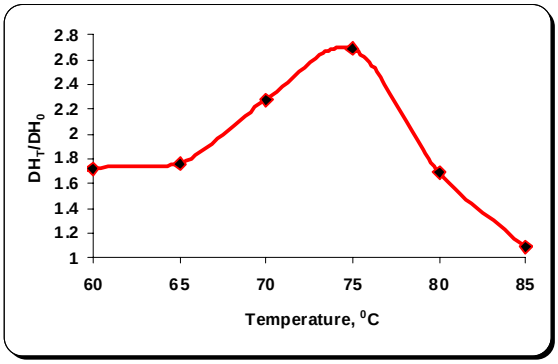

Figure 1. *Denaturation curve of WPC measured as susceptibility to Proteinase K hydrolysis after 10 minutes of holding at different constant temperatures*

When the pre-heated solutions were subsequently hydrolyzed by enzyme solutions an increase in susceptibility was observed in the temperature range of 60-75^{0}C (Figure 2).

The maximum extent of hydrolysis was observed after a preheating treatment at 75^{0}C. Longer treatment time resulted in a higher extent of susceptibility to proteolysis, until an equilibrium level was reached. The maximum value for DH was reached after 30 minutes at 75^{0}C, probably because at higher temperature-time combination, the molecular association is favoured.

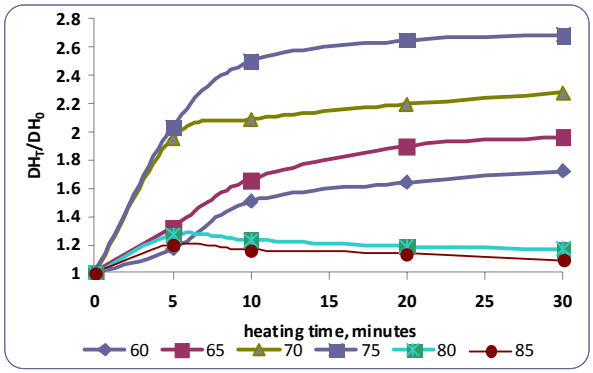

Figure 2. *Heat induced changes in susceptibility of WPC to Proteinase K hydrolysis*

Hydrolysis experiments were carried out at 7.5 and 37°C, by adding 100 µL of enzyme solution to 0.5 mL of 2.5 mg/ml WPC pre-heated solutions.

It can be seen from Figure 2 that at 80 and 85^{0}C the DH decreased which means that the protein molecules can be involved in polymerization via sulphydryl-disulfide interchange reactions and hydrophobic interactions as explained by Sava *et al.* (2005).

In the second set of experiments, the samples were heat treated at different temperature for 15 minutes and the hydrolysis was performed for 60 minutes. The results are given in Figure 3.

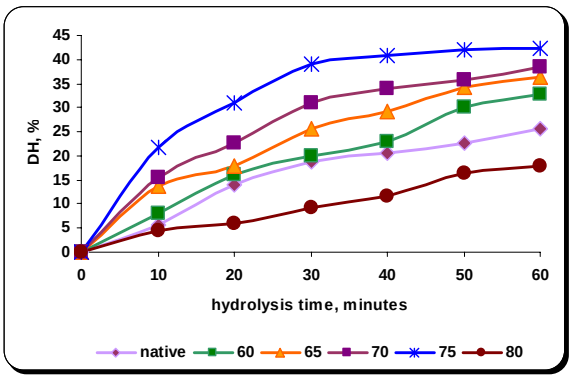

Figure 3. *The increase in DH % as a function of temperature and hydrolysis time*

Hydrolysis experiments were carried out at 8.0 and 37°C, by adding 100 µL of enzyme solution to 1 mL of 25 mg/ml WPC pre-heated solutions.

It can be seen that the DH increases with temperature in the temperature range of 60 to 75^{0}C and decreased at higher temperature. The maxim extent of hydrolysis was reached after a preheating treatment at 75^{0}C (DH% = 42.2 ± 2.3). At 80^{0}C, the DH values were lower even when compare with the native state.

This phenomenon is due to the fact that accessibility of the specific peptide bonds to the enzymes is enhanced, because of the unfolding of the protein molecules in lower temperature range studied. At temperatures higher than 75°C, the decrease in DH is probably due to the association/aggregation of the molecules, which can hide the specific peptides bonds for the enzymatic cleavage.

Protein structure is profoundly affected by environmental factors, as *p*H, temperature, protein concentration, etc. The protein conformation is influenced by several processes, such as rupture of non-covalent and covalent interactions that may be occurring simultaneously as the solution is heated and the *p*H is adjusted (Phillips *et al.*, 1994). It is possible that during heating at neutral and alkaline *p*H, the proteins, and especially β-LG to undergone critical conformational changes that result in exposure of enzymes sensitive peptide.

The susceptibility of WPC to enzymatic hydrolysis is of practical interest, due to the fact that whey proteins are the major ingredients for infant formula. The conformational changes of WPC induced by the thermal and enzymatic treatment are also associated with the improvement of the functionality of hydrolysates, thereby increasing the applications of the protein as a potential food ingredient. Ju *et al.* (1997) found a linear relation between DH% and the gel firming for the whey protein isolate that was denatured by heating at 80^0C for 2-30 min and hydrolysed with *Bacillus licheniformis*. Additionally, the hydrolysis of β-LG with trypsin is known to generate bioactive peptides and also peptides with emulsifying properties. As different final levels of DH10 after thermal treatment were observed, differences in solubility and foaming properties are also expected after a limited proteolysis.

Kinetics of heat-induced changes in enzymatic susceptibility of WPC at *p*H 7.5

The time-dependent changes in susceptibility of WPC to enzymatic hydrolysis by Proteinase K, are showed in Figure 2, for different thermal treatments. The increase in DH due to thermal treatment could be described by a first-order fractional conversion kinetic model in the temperature range of 60 to 75^0C. First-order kinetics is a consequence of a mechanism where the rate-controlling step is the first attack on the tertiary structure of the protein. The rate constants at different temperatures of the fractional conversion model for thermal denaturation of WPC as measured by DH are given in Table 1.

In the temperature range of 60 to 65°C, the influence of temperature is lower compared with higher temperature. This indicates that heating WPC solutions in this range induced possible reversible changes in proteins conformation. It can be seen that *k*-values for the increase in Proteinase K susceptibility of WPC at 75^0C are 3.5 times higher than those for 60^0C.

Based on equations 2, parity charts representing the correlation between predicted and experimental values were plotted, providing a good fit of the experimental data compared with the predicted data (Figure 4).

Table 1. *Kinetic parameters k and E_a estimated by the first order fractional conversion model describing heat-induced changes on the susceptibility of WPC to enzymatic hydrolysis at pH 7.5*

Temperature (°C)	**k (min^{-1})**
60	8.1±0.02[a]
65	9.3±0.015
70	19.85±0.021
75	28.4±0.039
E_a ($kJ.mol^{-1}$)	**86.4±16.0**

[a] : values ± standard errors of regression.

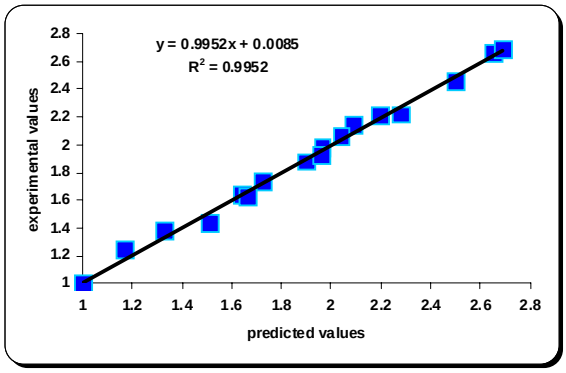

Figure 4. *Correlation between the predicted values for DH and the experimental values according to equation 2*

In the temperature range of 60-75°C, the temperature dependence of the rate constants could be described by the Arrhenius model (equation 3), resulting in an activation energy of 86.4±16.0 kJ/mol (r^2 = 0.93) (Figure 5).

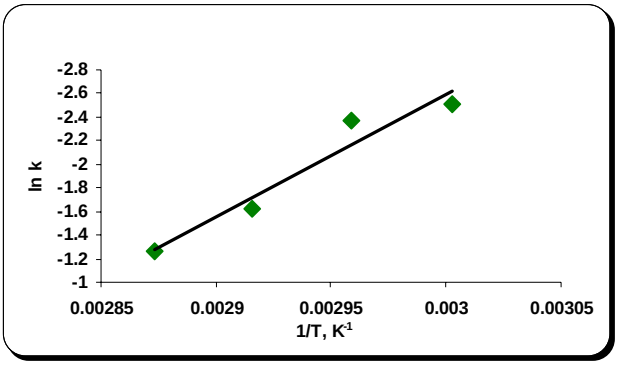

Figure 5. *Temperature dependence of the rate constants of the fractional conversion model*

Conclusions

Thermal treatment increased the extent of hydrolysis of WPC by Proteinase K. WPC seems to be more heat-sensitive for hydrolysis in the temperature range of 60 to 75^{0}C, confirming that the substrate nature is changing from dimmer to monomer when the *p*H is adjusted a neutral and alkaline values, with the exposure of hydrophobic residues. This phenomenon is due to the fact that accessibility of the specific peptide bonds to the enzymes is enhanced, because of the unfolding of the protein molecules. At temperatures higher than 80°C, the decrease in DH is probably due to the association/aggregation of the molecules, which can hide the specific peptides bonds for the enzymatic cleavage. This observation confirms that the highly reactive monomers formed during heat-treatment associate to form non-covalent/covalent aggregates.

These results indicate that the susceptibility of WPC to proteolysis can be modified by thermal treatment, and it can be important for modification of the functional properties of whey proteins as a potential ingredient in different food systems.

From another point of view, milk proteins are considered the most important source of bioactive peptides. The beneficial health effects may be attributed to numerous peptide sequences exhibiting antimicrobial, antioxidative, antithrombotic, antihypertensive, immunomodulatory and opioid activities, among others. Further studies are needed in order to identify and characterize the peptides formed after heat pre-treatment and enzymatic hydrolysis of WPC. These specific and complex issues are some of the main objectives of our Research Project PN-II-ID-PCE-2008-2, Idea, ID 517 – *Research resulting in analytical systems for Romanian milk and dairy products traceability in order to comply with European quality and safety criteria*.

Acknowledgement

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

BIOALIMENT Research Platform (www.bioaliment.ro) is also acknowledged for providing technical support.

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