THE INFLUENCE OF PROCESSING AND MEDIUM COMPOSITION ON THE THIOL AVAILABILITY OF WHEY PROTEIN CONCENTRATE

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Abstract. *Whey protein concentrates (WPC) play an important role in the development of functional foods with an increase in global demand. Whey proteins are highly soluble over a broad range of pH, a property that is important in their application as foaming, emulsifying, gelling, and water-binding agents in various types of food products.*
Denaturation of whey proteins is critical in modifying the functional behavior of dairy products. A knowledge of the thermal behavior of WPC occurring in different medium conditions, might allow to choose the most appropriate technology and to manipulate the thermal regime for a particular process or/and products, so that the final product possesses the optimal desirable properties.
The objective of this study was to investigate the heat induced changes in whey protein concentrate solution at pH 7.5 and 6.6 in salt conditions ($CaCl_2$) on thiol availability.
The results presented in this paper demonstrate that heat-induced changes in WPC greatly influence their degree in surface SH exposure. The exposure was more pronounced at higher temperatures. However, above 80°C, the formation of disulfide bonds seems to be favored, resulting in a lower level of surface reactive SH groups after prolonged heating compared with the lower treatment temperatures.
The increase was related to the intensity of applied temperature-time combination and medium composition. The thermal denaturation of WPC is regarded as a complex reaction involving several unfolding steps and ending in aggregation reactions.

Keywords*: heat-treatment, sulfhydryl groups, functional properties*

Introduction

Whey proteins are currently seen as a commodity product in food industry. They are largely used as emulsifiers, texture enhancers and healthy ingredients [1]. The functional properties of whey proteins may be classified into three main groups: (a) *hydration*, dependent upon protein-water interactions which have an important bearing on wetability, swelling, adhesion, dispersibility, solubility, viscosity, water absorption and water holding; (b) *interfacial,* including surface tension, emulsification and foaming characteristics; and (c) *aggregation and gelation*, which are related to protein-protein interactions [2]. Heat treatments are important steps in both production of whey protein concentrates and processing of food products. The commonly used heat treatments are preheating, pasteurization, and sterilization [3].

The functionality of the whey proteins is strongly influenced by the processing conditions and can be modified by thermal treatment either reversibly or irreversibly. The main components of whey proteins are β-lactoglobulin and α-lactalbumin.

When the whey proteins are heated denaturation of β-lactoglobulin occurs. This protein contains two intra-molecular disulphide bonds and one free SH-group (SH^{121}). During heat treatment these thiol groups are responsible for changes in the

structure and thus in functionality of whey proteins.
Knowledge in the thermal behaviour of WPC occurring in different medium conditions, might allow to choose the most appropriate technology and to manipulate the thermal regime for a particular process or/and products, so that the final product possesses the optimal desirable properties.

Materials and methods

WPC was purchased from KUK Romania. The chemicals 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecylsulfate (SDS) were obtained from Sigma Chemicals (St. Louis, MO, USA). All solvents and chemical reagents were of analytical grade.

Isothermal Treatment of WPC solutions
5 mL of WPC solutions (2.5% in distilled water at two pH values: 7.5 and 6.6 with or whitout 0.002 M $CaCl_2$) were heated in glass tubes in a thermostatically controlled water bath at constant temperatures between 70 and 85°C for 0 to 30 min. After the heat treatment, the samples were immediately transferred to ice-cold water to prevent further denaturation. The samples were stored at 4^0C over night.

Thiol availability
The exposed SH groups were determined as described by Sava et al. (2005) [4]. The (un) heated samples (50 µL) were diluted with appropriate buffer solution (standard solution at pH 8.0 for exposed SH groups. DTNB reagent (10 µl) was added to the (un)treated samples. The absorbance at 412 nm was measured against a reagent blank after 15 min at 20°C.
The SH groups (µmol/g protein) were calculated with the equation 1:

$$\mu\text{mol SH/g protein} = \frac{(A_1 - A_0) \cdot D}{C \cdot 13600} \quad \textbf{(1)}$$

where A_1 is the absorbance of (un)heated sample at 412 nm, A_0 is the absorbance of the blank , d the dilution factor, 13 600 the molar extinction coefficient, and c the protein concentration (mg/mL).

Results and discussion

The influence of pH
In the first sets of experiments, the samples were heat-treated at two different pH-values: 6.6. and 7.5.
Figure 1 shows the extent of SH groups exposure at pH 6.6. As it can be seen, the maximum exposure was reached after 20 minutes of keeping at 85^0C.

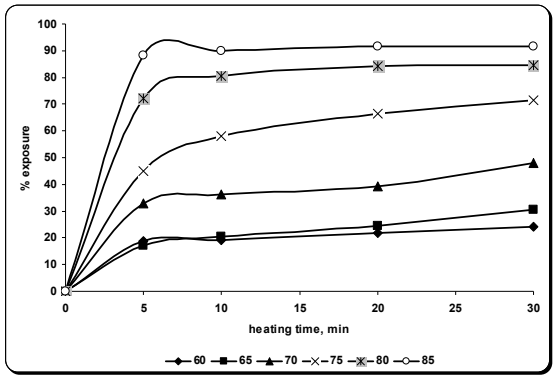


Figure 1. The extent of SH groups exposure (%) after heat-treatment of WPC solutions at pH 6.6

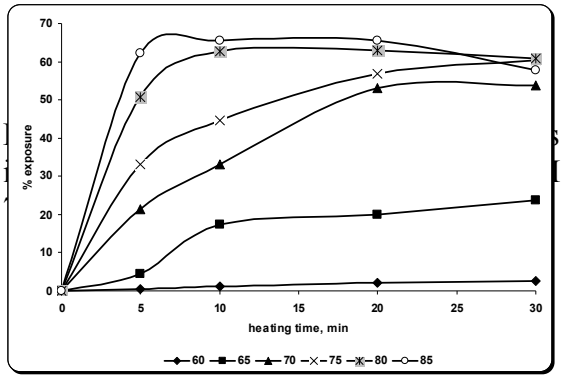


Figure 2. The extent of SH groups exposure (%) after heat-treatment of WPC solutions at pH 7.5

Heating the samples at pH 7.5 caused the exposure of free SH groups, the maximum extent was measured at 85^0C after 10 minutes of keeping.

At both pH values studied, the increase of keeping time resulted in a gradual increase in the extent of free SH groups exposure. These heat-induced changes in free-SH complied with observations made by Sava et al., (2005) [4].

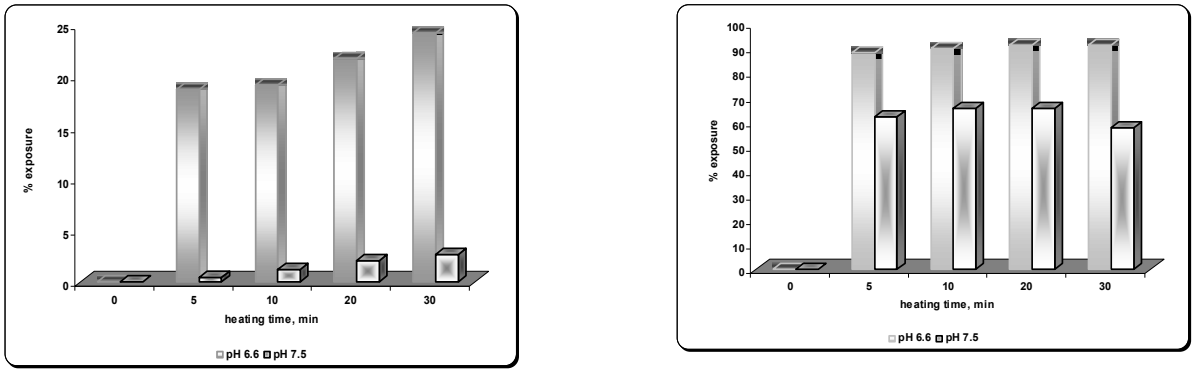
One can see from **figure 3**, that at pH 7.5 the extent of denaturation, expressed as exposure of SH groups is considerable lower as compared to pH 6.6.

The reactivity of free thiol group in β-lactoglobulin is strongly dependent on pH.

β-lactoglobulin undergoes pH-dependent conformational changes in the pH range of 6.5 to pH 7.5, the so called N→R or Tanford transition [5]. These changes are associated with an anomalous carboxylate residue, which has a p*Ka* of 7.3 instead of 4.5, and which was tentatively identified as Glu^{89} [6].

According to this theory, the dimmer starts to dissociate, followed by critical conformational changes of the molecule with the exposure of hydrophobic groups and reactive free sulfhydryl group, known as the Tanford transition [7].

Figure 3 shows the comparative analysis of % exposure at 60^0C and 85^0C at both pH values.



a) **b)**

Figure 3. The degree of free SH groups exposure at 60^0C (a) and 85^0C (b)

Under sub-denaturation condition, the degree of exposure was considerable higher at pH 6.6 (figure 3, a) when compared to pH 7.5.

It seems that when protein thiol availability is measured close to the isoelectric point of proteins, the isoelectric precipitation is stimulated. This affirmation is sustained by the increase in the turbidity and decrease of solubility of the samples at both pH values studied (data not shown).

When the temperature is increased, the free SH groups become more accessible for interaction with DTNB at both pH values.

As it was mentioned earlier, the heating of whey proteins lead firstly to the denaturation of the β-lactoglobulin. This denaturation process involves a rearrangement of the tertiary structure so that the free thiol group from cysteine 121, in the native state buried within the protein molecule, now becomes accessible. This activated thiol group can subsequently react with disulfide bonds that are also present in β-lactoglobulin or in other whey proteins such as α-lactalbumin in an exchange (or propagation) reaction, or can react with another thiol group to form a disulfide bond (termination reaction), as it was explained by Roefs and De Kruif (1994) [8].

Influence of salt

The presence of calcium enhances the heat-induced aggregation of WPC [9].

The formation of whey protein aggregates can be affected by the addition of salts. In this study, the solutions at pH 6.6 and 7.5 were heat-treated in the presence of 0.002 M $CaCl_2$.

Figure 4 shows the heat-induced changes in free-SH groups exposure at pH 6.6 in the presence of 0.002 M $CaCl_2$.

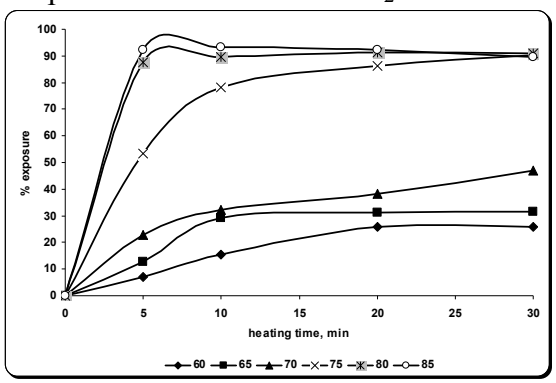


Figure 4. The extent of SH groups exposure (%) after heat-treatment of WPC solutions at pH 6.6 in the presence of 0.002 M $CaCl_2$

Despite the intensity of heat treatment, the exposure of free SH groups remained high, the maximum extent of denaturation was reached after 20 minutes at 85^0C (91.5% ± 0.9).

Figure 5 shows the extent of WPC denaturation at pH 7.5 in the presence of the same concentration of salt.

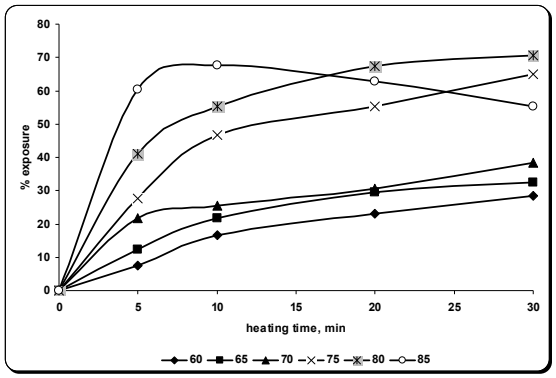
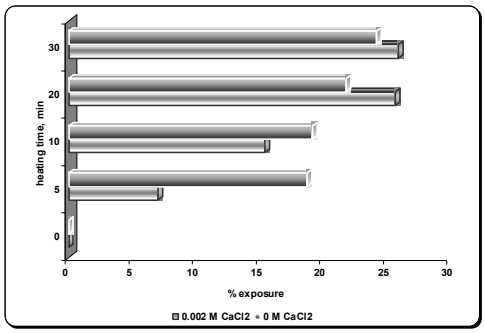


Figure 5. The extent of SH groups exposure (%) after heat-treatment of WPC solutions at pH 7.5 in the presence of 0.002 M $CaCl_2$

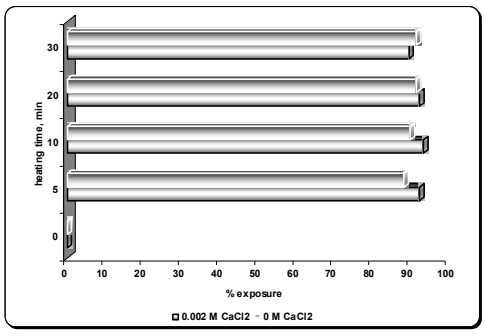
The increase in SH groups exposure was dependent on the intensity of applied thermal treatment and the duration of the treatment, and consequently, for both sets of experiments, was significantly pronounced at higher temperature. The heating at pH 7.5 in the presence of salt leads to a lower extent of exposure, the maximum extent was reached at 85^0C after 10 minutes of heating. It can also be seen from figure 5 that under more severe treatment conditions, the content of free exposed SH groups decrease, probably because the electrostatic interactions are favoured.

Figure 6 shows the degree of free SH groups exposure at pH 6.6 at 60^0C (a) and 85^0C (b) in the presence of 0.002 M $CaCl_2$.

The addition of salt at 60^0C caused no denaturation in the first 10 minutes of heating, with a slight increase in exposure after 20-30 minutes of keeping.



(a)



(b)

Figure 6. The degree of free SH groups exposure at pH 6.6 after heat treatment at 60^0C (a) and 85^0C (b)

The presence of calcium under more severe heat treatment conditions did not have any influence on the free SH exposure for both pH values studied.
There was a significant difference in the degree of exposure when samples at pH 7.5 were heated at 60^0C in the presence of $CaCl_2$ (**figure 7**). The degree of exposure was almost 11 times higher after 30 minutes of keeping in the presence of salt.

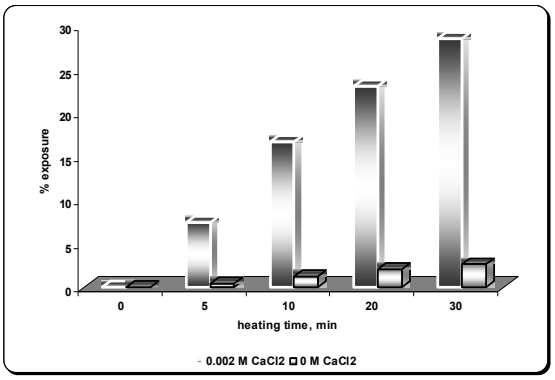


Figure 7. The degree of free SH groups exposure at pH 7.5 after heat treatment at 60^0C with and without salt addition

This observation indicates that thermal denaturation of WPC as measured by the changes in surface SH groups exposure involves 2 steps: an unfolding step (60 to 75°C) and an aggregation one (75 to 85°C), that mostly follows unfolding.
It has been suggested that three effects, or a combination of them, might be responsible for calcium-induced protein aggregation [10]. The first phenomenon is related to intermolecular cross-linking of adjacent negatively charged or carboxylic groups by the formation of protein-Ca^{2+}-protein complexes [11]. The second phenomenon is the intra-molecular electrostatic shielding of negative charges on the protein [12]. Monovalent and divalent cations both screen electrostatic interactions between charged protein molecules although the effect is greater with divalent cations [13]. The third phenomenon is an ion-induced conformational change, which leads to altered hydrophobic interactions and aggregation at elevated temperatures [9].

Conclusions

The results presented in this paper demonstrate that heat-induced changes in WPC greatly influence their degree in surface SH exposure. The increase was related to the intensity of applied temperature-time combination and medium composition.
The exposure of surface to SH groups was more pronounced at pH 6.6, especially in a higher temperature range. These behaviours may be explained by the fact that in lower temperature range the proteins firstly unfold, as shown by the time-and temperature-dependent increases in the surface SH groups and then aggregates at higher temperature.
At pH 6.6, in a lower temperature range, the addition of $CaCl_2$ did not have any influence on the degree of exposure at the surface of molecules. At pH 7.5, the calcium ions had significantly higher effect on the SH groups exposure to the temperature range of 60 to 75^0C.
The exposure was more pronounced at higher temperatures. However, above 80°C, the formation of disulfide bonds seems to be favoured, resulting in a lower level of surface reactive SH groups after prolonged heating compared to the lower treatment temperatures.
The thermal denaturation of WPC is regarded as a complex reaction involving several unfolding steps and leading to aggregation reactions.
It should be mentioned that the phenomena taking place in the present model system do not necessarily predict the behaviour of WPC in commercial dairy foods, since the presence of other components (sugars, fats, other proteins, etc.) could modify the observed behaviour.
Further research is underway to optimize the conditions of thermal treatment, in

order to obtain enhanced functional properties related to protein-water and protein-protein interactions.

Acknowledgment

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