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# EFFECTS OF pH ON THE SURFACE HYDROPHOBICITY OF PROTEINS FORMING AN ENZYMATIC GEL NETWORK IN UNHEATED AND HEATED SOLUTIONS OF MILK PROTEIN CONCENTRATES

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The objective of the present study was to compare changes in the surface hydrophobicity of proteins forming a rennet gel network at pH 6.6 and 6.0. The substrates were unheated and heated (72°C/15 s; 92°C/60 s) aqueous solutions of milk protein concentrate (protein concentration 3.3%). The contents of nitrogen, calcium and ANS (nmol ANS/mg protein) bounded to protein particles sedimented during ultracentrifugation of curds and substrates (insoluble fractions after ultracentrifugation (110 000 g, 1 hour) were determined.

There were no significant differences in the calcium to protein ratio in the curd matrices obtained from unheated and heated (72°C/15 s; 92°C/60 s) substrates. At pH 6.6 the index of ANS binding by insoluble proteins after curd ultracentrifugation was by 1.3%, 3.5% and 2.3% lower, compared with the corresponding substrate fractions. This suggests that not only hydrophobic domains of paracasein, formed during the enzymatic phase of coagulation, were used for gel network formation.

At pH 6.0 significant differences ($p=0.05$) between the indices of surface hydrophobicity of insoluble proteins after ultracentrifugation of curds and substrates were observed only in the case of concentrate solutions heated at 92°C ($\Delta$ANS = 0.14 nmol/mg protein), which may indicate a lower level of utilization of hydrophobic domains formed after proteolysis of κ–casein.

## INTRODUCTION

Hydrophobic interactions play a very important role in the formation of enzymatic and acid curds [Horne & Davidson, 1993]. Aromatic hydrophobicity is associated with the interactions of aromatic amino acid residues, whereas aliphatic hydrophobicity concerns aliphatic residues of amino acids. Higher significance is attributed to aromatic hydrophobicity, which – combined with the potential $\zeta$ – produces a considerable effect on protein solubility [Hayakawa & Nakai, 1985].

The aromatic hydrophobicity of proteins is usually measured by fluorometric methods, using 8-anilino-naphthalene-1-sulfonate (ANS). Numerous studies show that the number of hydrophobic sites on the protein surface changes as a result of milk heating and storage [Bonomi *et al.*, 1988]. It was found that an increase in surface hydrophobicity of all milk proteins is caused mostly by changes in the dominant casein, which may also be affected by interactions between κ–casein and $\beta$-lg [Carbonaro *et al.*, 1996].

Approximately 95% of milk casein is present in the form of micelles, *i.e.* a casein-phosphate-calcium complex. They may be separated by ultracentrifugation (110 000 g/1 h). The amount of ANS bounded by micelles per protein unit reflects the possibility of occurrence of hydrophobic interactions between these macromolecules. In the process of enzymatic coagulation, during hydrolysis of κ–casein, there appear additional hydrophobic domains, determining micelle association [Dziuba & Muzińska, 1998]. Hydrophobic interactions and calcium ions, incorporated into the gel network during coagulation, neutralizing the charge and forming calcium bridges, are two major factors responsible for curd formation and its further transformations [Horne & Davidson, 1993; Peri *et al.,* 1990].

The composition and structure of milk casein micelles may undergo various modifications during the production of spray-dried protein retentate and its utilization in different technological processes. These modifications determine changes in the functional properties. They result from changes caused by both heating (*i.e.* addition of part of denatured whey proteins to micellar casein) and disturbances of the dynamic equilibrium between the micellar and soluble fractions of milk, observed during ultrafiltration or pH lowering [Żbikowska & Szerszunowicz, 2003 a, b].

The objective of the present study was to compare changes in the surface hydrophobicity of proteins forming a rennet gel network at pH 6.6 and 6.0 in unheated and heated (72°C/15 s; 92°C/60 s) solutions of milk protein concentrates. It was assumed that insoluble fractions (110 000 g, 1 h) represent modified micelles in concentrate solutions, and proteins forming the gel matrix in enzymatic curds.

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## MATERIALS AND METHODS

**Materials.** Spray-dried skim milk retentate, known under the trade name "milk protein concentrate" (69.2% protein, 16.2% lactose, 4.2% water, 7.2% ash, 3.2% fat) was used in the experiment, along with chymosin (EC 3.4.23.4) [Sigma Chemical Company, cat. no. 7751, enzyme activity 23.5 U/mg protein] and ANS (8-anilino-naphthalene-1-sulfonate) [Sigma Chemical Company, cat. no. A-1028].

**Experimental.** Solutions of protein concentrate with pH 7.1 were prepared. After heating at 72°C/15 s and 92°C/60 s, the pH of the solutions was adjusted to 6.6 and 6.0 (BECKMAN Φ720 pH-meter) adding 4.4 mol/L lactic acid, and protein concentration was brought to a level of 3.3%, adding deionized water. The substrates were preserved with sodium azide and streptomycin (0.02 %), and stabilized at 8°C for 12 h, and then at 20°C for 2 h. Enzymatic curds obtained from substrates with the addition of 0.2 mmol ANS and chymosin (pH 6.6 – 1.07 x $10^{-6}$ g/mL, pH 6.0 – 0.40 x $10^{-6}$ g/mL), after 1.5-h incubation enabling to complete the enzymatic phase of coagulation, and substrates with the addition of 0.2 mmol ANS, were ultracentrifuged (110 000 g, 1 h, temp. 20°C) [centrifuge OTD COMBI, Sorvall Instrument, DuPont, rotor T-865, test-tube capacity 11.5 mL].

**Analyses.** The contents of nitrogen [Budsławski & Drabent, 1972] and ANS – by the fluorescence method [Peri *et al.*, 1990] were determined in the soluble fractions after ultracentrifugation. Fluorescence intensity was measured at a wavelength $\lambda$=480 nm, after excitation at $\lambda$=390 nm in the PERKIN ELMER LS 50 spectrofluorimeter. The amounts of nitrogen and ANS in the insoluble fractions were calculated as the difference between their total contents and contents in the soluble fractions. The index of surface hydrophobicity of proteins in the insoluble fractions was the amount of ANS bounded to them per mg protein (N x 6.38). The index of changes in surface hydrophobicity during curd formation was the difference between the amount of ANS bounded to the curd matrix and its amount bounded to modified micelles or protein macromolecules sedimented during ultracentrifugation in substrates (assuming that they form the gel matrix).

Calcium content was determined by atomic absorption spectrophotometry (UNICAM 939 Solar apparatus, Great Britain), after wet mineralization of samples [Whiteside & Miner, 1984], in the insoluble fractions at 110 000 g obtained after ultracentrifugation of solutions of milk protein concentrates and enzymatic curds.

**Analysis of results.** All analyses were made in three repetitions. The results are presented as means. SEM (standard error of mean) and significance of differences (p=0.05) were determined. A statistical analysis was performed with STATISTICA PL software.

## RESULTS AND DISCUSSION

### Effects of pH on differences between the hydrophobicity indices of insoluble proteins after ultracentrifugation of unheated and heated milk concentrate solutions

#### pH 6.6

At pH 6.6 the index of surface hydrophobicity of proteins of the insoluble fraction in substrates heated at 72°C/15 s was by 3.4% lower, compared with unheated substrates (Table 1). There were no significant differences between unheated substrates and substrates heated at 92°C/60 s. A higher w/w calcium to protein ratio in heated substrates (Table 1) confirms its role in charge neutralization, facilitating hydrophobic protein association and limiting the exposure of hydrophobic residues of amino acids. The results of the present and previous [Żbikowska & Szerszunowicz, 2003b] experiments show that a crucial factor is the presence of denatured whey proteins, which may prevent hydrophobic interactions between casein polypeptide chains in proteins of the insoluble fraction, but do not constitute a barrier for small molecules of ANS contained in the solvent.

TABLE 1. Protein content, Ca/protein ratio (w/w) and index of surface hydrophobicity of proteins in insoluble fractions after ultracentrifugation of unheated and heated solutions of milk protein concentrate (n=3).

| pH | Heating conditions | Protein (N x 6.38) (mg/mL) | Ca/protein (x $10^{-2}$) (w/w) | Index of surface hydrophobicity of proteins (nmol ANS/mg protein) |
|---|---|---|---|---|
| | - | $28.90^{a}$±0.06 | $2.43^{b}$±0.01 | $5.57^{a}$±0.01 |
| 6.6 | 72°C/15 s | $28.54^{b}$±0.02 | $2.56^{ab}$±0.00 | $5.38^{b}$±0.01 |
| | 92°C/60 s | $28.26^{c}$±0.00 | $2.62^{a}$±0.00 | $5.59^{a}$±0.00 |
| | - | $29.05^{b}$±0.02 | $1.94^{a}$±0.01 | $5.71^{a}$±0.00 |
| 6.0 | 72°C/15 s | $28.99^{b}$±0.02 | $1.96^{a}$±0.01 | $5.65^{ab}$±0.00 |
| | 92°C/60 s | $29.35^{a}$±0.00 | $2.14^{b}$±0.01 | $5.53^{b}$±0.01 |

The letters (a, b, c) denote the lack of significant differences (p=0.05) between the contents of particular components in unheated and heated (72°C/15 s; 92°C/60 s) solutions, separately for each pH level analyzed.

#### pH 6.0

At pH 6.0 significant differences were noted between the amount of ANS bounded by proteins of the insoluble fraction in unheated substrates and substrates heated at 92°C (Table 1). The hydrophobicity index in heated substrates was by 3.2% lower. Similarly as at pH 6.6, a higher calcium to protein ratio was recorded in the insoluble fractions of heated substrates, which confirms its role in facilitating hydrophobic interactions, leading to an increase in the protein content of insoluble fractions, and limiting the access of the marker to the aromatic residues of amino acids.

### Effects of pH on differences between the hydrophobicity indices of insoluble proteins after ultracentrifugation of the substrates analyzed and enzymatic curds obtained from them

The index of ANS binding in the insoluble protein fractions after ultracentrifugation of enzymatic curds obtained at pH 6.6 was lower than in the insoluble fractions of substrates (Tables 1 and 2). This indicates a higher, compared with milk, contribution of hydrophobic interactions to curd matrix formation [Dziuba & Muzińska, 1998], and suggests that not only hydrophobic domains of paracasein, formed during the enzymatic phase of coagulation, were used for gel network formation.

TABLE 2. Protein content, Ca/protein ratio (w/w) and index of surface hydrophobicity of proteins in insoluble fractions after ultracentrifugation of enzymatic curds obtained from the substrates analyzed (n=3).

| pH | Heating conditions | Protein (N x 6.38) (mg/mL) | Ca/protein (x $10^{-2}$) (w/w) | Index of surface hydrophobicity of proteins (nmol ANS/mg protein) |
|---|---|---|---|---|
| | - | $30.88^{b}\pm0.00$ | $2.69^{a}\pm0.01$ | $5.50^{a}\pm0.00$ |
| 6.6 | 72°C/15 s | $31.03^{a}\pm0.02$ | $2.67^{a}\pm0.01$ | $5.19^{b}\pm0.00$ |
| | 92°C/60 s | $31.05^{a}\pm0.02$ | $2.71^{a}\pm0.02$ | $5.46^{a}\pm0.00$ |
| | - | $30.88^{a}\pm0.00$ | $2.11^{a}\pm0.01$ | $5.69^{a}\pm0.00$ |
| 6.0 | 72°C/15 s | $30.94^{a}\pm0.00$ | $2.13^{a}\pm0.01$ | $5.68^{a}\pm0.00$ |
| | 92°C/60 s | $30.94^{a}\pm0.00$ | $2.07^{a}\pm0.02$ | $5.67^{a}\pm0.00$ |

Explanations as in Table 1.

During curd formation in unheated substrates, the hydrophobic domains participated to a lower, and calcium to a higher degree in gel network formation (ΔCa/protein), compared with heated substrates (Figures 1 and 2). In contrast to the insoluble fractions of substrates, no significant differences in the calcium to protein ratio were found in the curd matrices obtained from unheated and heated substrates (Tables 1 and 2). This suggests that at pH 6.6 a similar calcium to protein ratio is necessary for curd matrix formation both in heated and unheated substrates. It follows that an increase in the concentration of denatured whey proteins in the curd matrix may affect a lower contribution of calcium to its formation.

At pH 6.0 significant differences between the indices of surface hydrophobicity of insoluble proteins after ultracentrifugation of curds and substrates were observed only in the case of concentrate solutions heated at 92°C (Figure 1), where the amount of ANS per mg protein was higher in curd matrices than in proteins of the insoluble fraction of substrates. This may indicate a lower level of utilization of hydrophobic domains formed after proteolysis of κ–casein, a lower contribution of calcium (Figure 2), compared with unheated substrates and substrates heated at 72°C, and a higher contribution of denatured whey proteins [Żbikowska & Szerszunowicz, 2003b] to gel matrix formation.

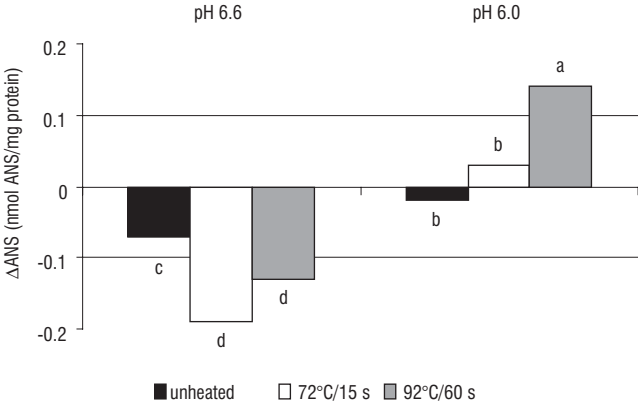


FIGURE 1. Effect of proteolysis pH on changes in the surface hydrophobicity index during curd formation. ΔANS – difference between the index of surface hydrophobicity (nmol ANS/mg protein) of proteins of the insoluble fractions after curd ultracentrifugation (after the enzymatic phase) and substrates (before proteolysis). The same letters denote the lack of significant differences (p=0.05).

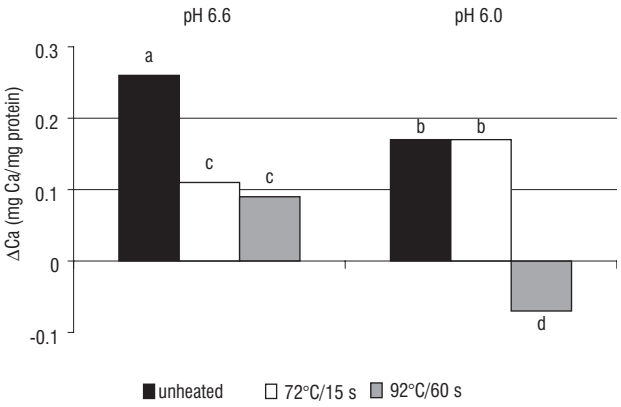


FIGURE 2. Contribution of Ca to gel network formation at pH 6.6 and 6.0 in heated and unheated concentrate solutions. ΔCa – difference between the Ca content (mg Ca/mg protein) of the insoluble fractions after curd ultracentrifugation (after the enzymatic phase) and substrates (before proteolysis). The same letters denote the lack of significant differences (p=0.05).

β–Casein losses, observed in previous investigations, may also be of primary importance [Żbikowska & Szerszunowicz, 2003a].

## CONCLUSIONS

1. The level of hydrophobic domain utilization and calcium contribution to rennet gel network formation in unheated concentrate solutions were higher at pH 6.6 than at pH 6.0 (p=0.05).

2. During rennet gel network formation the level of hydrophobic domain utilization was higher at pH 6.6 (p=0.05) and lower at pH 6.0 in heated substrates, compared with unheated substrates. At pH 6.6 it was connected with a lower contribution of calcium to rennet gel network formation and a higher degree (p=0.05) of incorporation of non-micellar proteins to the gel network, whereas at pH 6.0 – with a higher participation of calcium in the formation of this network.

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## WPŁYW pH NA HYDROFOBOWOŚĆ POWIERZCHNIOWĄ BIAŁEK TWORZĄCYCH SIEĆ ŻELU ENZYMATYCZNEGO W NIEOGRZEWANYCH I OGRZEWANYCH ROZTWORACH KONCENTRATU BIAŁEK MLEKA

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Celem badań było porównanie zmian hydrofobowości powierzchniowej białek tworzących sieć żelu podpuszczkowego przy pH 6.6 i 6.0. Substratem były nieogrzewane i ogrzewane (72°C/15 s; 92°C/60 s) wodne roztwory koncentratu białek mleka o stężeniu białka 3.3%. Oznaczono zawartość azotu, wapnia oraz ilość ANS (nmol ANS/mg białka) związanego z cząstkami białek osadzającymi się podczas ultrawirowania skrzepów i substratów (frakcje nierozpuszczalne po ultrawirowaniu (110 000 g, 1 godz.)).

W matrycach skrzepów uzyskanych z substratów nieogrzewanych i ogrzewanych (72°C/15 s; 92°C/60 s) nie stwierdzono istotnych różnic pomiędzy stosunkiem Ca/białka (tab. 2). Przy pH 6.6 mniejszy o 1.3%, 3.5% i 2.3% wskaźnik wiązania ANS przez białka nierozpuszczalne po ultawirowaniu skrzepów, w porównaniu do analogicznych frakcji substratów pozwala przypuszczać, że podczas tworzenia sieci żelu wykorzystane zostały nie tylko domeny hydrofobowe kazeiny-para-$\kappa$ utworzone podczas enzymatycznej fazy koagulacji.

Przy pH 6.0 istotne różnice ($p=0.05$) pomiędzy wskaźnikami hydrofobowości powierzchniowej białek nierozpuszczalnych po ultrawirowaniu skrzepów i substratów zaobserwowano jedynie w przypadku roztworów koncentratu ogrzewanych w 92°C ($\Delta$ANS = 0.14 nmol/mg białka), co może wskazywać na mniejsze wykorzystanie domen hydrofobowych tworzących się po proteolizie kazeiny-$\kappa$.