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EFFECT OF TEMPERATURE ON IMMUNOREACTIVE PROPERTIES OF COW MILK WHEY PROTEIN. I. α-LACTALBUMIN

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The effect of heat induced changes on the immunoreactivity of α-lactalbumin (α-la) in whey was evaluated. Whey and acidified α-la solutions were heated for various times at temperatures between 60 and 100 °C. After heating, the samples were clarified and the protein content (by Bradford's method), the quantity of α-la (by FPLC method) and the immune response to an anti α-la probe were determined. The samples heated at 80 °C were characterized by the highest α-la immunoreactivity properties. Raising temperature up to 90 °C and 100 °C caused a marked decrease in α-la immunoreactivity. As was found by FPLC method in the samples heated at 60 and 65 °C, α-la content changes were not statistically significant. A gradual decrease in the content of α-la was observed with increased temperature and duration of heating.

Keywords: α-lactalbumin, ELISA, heat treatment

Heat treatment (i.e. thermization, pasteurization, sterilization, UHT) is commonly applied in the dairy industry as it can effectively prolong the shelf life of dairy products (CARBONARO et al., 1997). It is also used during various manufacturing processes to produce the desired properties of products (e.g. texture of yoghurt) (DANNENBERG & KESSLER, 1988; PARAF et al., 1991). It can also modify the properties of products, making it possible to obtain a product of improved quality and with reduced allergenic properties. Heat treatment may induce changes in protein structure and thus increase or decrease the antigenicity of the protein.

Whey proteins are important constituents of milk proteins due to their high content of essential – including a number of sulphuric – amino acids. Milk proteins (β-lactoglobulin, caseins) are regarded as very common allergens. α-Lactalbumin has not been universally defined as an allergen, however there are reports in which α-la has been identified as one of the allergens of milk (KING, 1976; BAHNA & HEINER, 1980). Heat treatment is known to cause protein denaturation, which changes its structure and can significantly modify its immunoreactive properties. It has been found that the denaturation degree of α-lactalbumin and β-lactoglobulin proteins increases with increasing temperature and the duration of heat treatment, and that a part of denatured

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whey proteins can associate with casein micelles – through disulphide bonds or hydrophobic interaction – as casein precipitates at its isoelectric point (pH 4.6) (TOWNEND & GYURICSEK, 1973; DANNENBERG & KESSLER, 1988; LAW et al., 1993). The changes in the content of particular protein components can be controlled by means of chromatographic and electrophoretic methods such as ion exchange chromatography (ANDREWS et al., 1985; LAEZZA et al., 1991), reversed phase high performance liquid chromatography (PARRIS et al., 1977; PARRIS, 1989) and capillary electrophoresis (RECIO et al., 1995; 1996; MIERZEJEWSKA et al., 2002).

The aim of this work was to examine the effect of heat treatment parameters (temperature and time) on the immunoreactive properties of α-lactalbumin in whey and in a model solution.

1. Material and methods

1.1. Raw whey

Whey was obtained from skimmed cow milk following the precipitation of casein with 1M HCl at pH 4.6, and centrifugation at 12 000×*g* for 15 min (BASCH et al., 1985; MORR, 1990; LAW et al., 1993).

1.2. Heating of whey

Raw whey (10 ml) was measured into 50 ml conical flasks. The flasks were sealed and shaken in a HS-B 20 water bath (IKA LABORTECHNIK, Warszawa, Poland) at temperatures of 60, 65, 70, 72, 80, 90 and 100 °C. The samples taken for the analysis after 15 and 30 s, and 1, 5, 10 and 30 min, were cooled in an ice bath, divided into two lots and stored in frozen form (–20 °C) until being analysed. After thawing, the samples were re-centrifuged at 10 000×*g*.

Additionally, a model experiment was also carried out. Pure α-la (L 6010, Sigma, Poznan, Poland) (2 mg ml^{-1}) dissolved in water with pH adjusted to 4.6 was heated under the same conditions as the whey samples.

1.3. Sample preparation

The whey samples were centrifuged at 12 000×*g* for 30 min and filtered through filters with a pore diameter of 0.22 μm. They were then applied to the HR 5/5 column (Pharmacia, Warszawa, Poland) filled with WP PEI ion exchanger (Bakerbond, Lodz, Poland) at a flow rate of 0.5 ml min^{-1} (ANDREWS et al., 1985; PARRIS, 1989; LAW et al., 1993).

1.4. Protein determination

Protein content in the samples was determined according to the method of BRADFORD (1976). Bovine serum albumin (A 4503, Sigma, Poznan, Poland) was used as a standard protein in the range of 10–100 μg ml^{-1}.

1.5. Antibodies production

Antibodies were produced by three rabbits. Immunogen prepared for the first immunization contained 0.5 ml of antigene (α-la, L 6010, Sigma, Poznan, Poland) solution in 0.9% sodium chloride (2.0 mg ml^{-1}) emulsified with equivalent volume of Freud's complete adjuvant (F 5881, Sigma, Poznan, Poland) (BEAN, 2001). Next 3 immunizations were made then at monthly intervals in the presence of Freud's incomplete adjuvant (F 5506) and the last two immunizations were without adjuvant with the same volumes and concentrations of antigene as described previously. All immunization injections were given subcutaneously (two sites) and intramuscularly (one site) (0.25 ml per site) (HITZELBERG et al., 1985; HANLEY et al., 1995). Production of antibodies and increase in their titre were controlled using the indirect ELISA method by taking blood samples from the marginal vein of rabbit 2–3 days prior to the subsequent scheduled immunization (WRÓBLEWSKA & JĘDRYCHOWSKI, 1994).

Ten days after the last immunization the rabbits were exsanguinated. Blood was incubated for 1 h at 30 °C. Following centrifugation at 1500×*g* for 20 min, serum IgG antibodies were obtained at 20% saturation with sodium sulphate (GARVEY et al., 1977). After centrifugation at 1500×*g* for 30 min, the pellet was dissolved in phosphate buffer of pH 8.8, dialysed for 15 h at 4 °C with phosphate buffer being changed four times and the IgG fraction thus obtained was lyophylised.

1.6. ELISA method

Competitive ELISA was used to determine the immunoreactivity of whey samples during heating. The microtitre plate (Nunc®) was coated with 100 μl/well of pure antigen (α-la, L 6010, Sigma, Poznan, Poland) in 50 mM – carbonate buffer of pH 9.8 (10 μg ml^{-1}), and incubated for 12–18 h at 4 °C. The plate was then washed four times with 10 mM phosphate buffer of pH 7.4, containing 0.5% Tween 20. This washing system was used after each analytical step. Residual free binding sites were blocked with 150 μl/well of 1.5% gelatin (G 9382, Sigma, Poznan, Poland) in coating buffer for 30 min at 25 °C. Next, the solution of rabbit antibodies (50 μl/well), diluted 1:12000 with PBS and the sample examined (50 μl/well) were added at the same time to the antigen-coated and gelatin blocked well. The plate was then incubated for 1 h at 37 °C. After washing, the plate was incubated for 1 h at 37 °C with 100 μl/well of peroxidase-conjugated goat anti-rabbit immunoglobulin (A 6154, Sigma, Poznan, Poland), followed by washing and addition of 100 μl/well of *o*-phenylene-diamine dihydrochloride (P 8287, Sigma, Poznan, Poland) in 9 mM citrate buffer of pH 5.0 (100 μl/well). After incubation for 30 min, 100 μl/well of 4 M sulphuric acid was added to stop the reaction (HEPPELL, 1985; TIJSEN, 1985; KARASOVÁ et al., 1999).

Absorbance was read at 492 nm on an automatic plate reader (Reader 510, Organon Teknika, Brussels, Belgium). The results were statistically evaluated by the use of Immunofit™ EIA/RIA software (Beckman Inc., Warszawa, Poland). The α-lactalbumin immunoreactivity was calculated from the standard curve (α-lactalbumin concentrations ranged from 0.001 to 150 μg ml^{-1}) and expressed in μg ml^{-1}. All analyses were performed in triplicate. The results were expressed as mean value and standard deviation.

1.7. FPLC chromatography

The chromatographic separation was performed in the linear gradient of two buffers: A: 0.1 M Tris/HCl, pH 7.0 and B: 0.1 M Tris/HCl, pH 7.0 containing 1 M NaCl. Elution profile was monitored by absorbance at 280 nm. The FPLC system (Pharmacia, Warszawa, Poland) was programmed by the use of FPLC Manager software (v.3.1) (Pharmacia, Warszawa, Poland). α-la content was expressed as percentage, assuming that the particular protein content was proportional to the peak area corresponding to the standard. Pure α-la (L 6010, Sigma, Poznan, Poland) was used as standard. The results were expressed as mean value and standard deviation.

2. Results and discussion

In the present work the cow milk whey was heated at 60, 65, 70, 72, 80, 90 and 100 °C for 0.25, 0.5, 1, 5, 10 and 30 min. The α-lactalbumin content of the whey samples thus obtained was analyzed for immunoreactivity changes. In addition, an experiment in a model system was also performed in order to exclude the effect of environmental factors (i.e., proteins other than those examined) on the behaviour and immunoreactivity of α-lactalbumin during heating.

Changes in the immunoreactive properties of whey α-lactalbumin depending on the process conditions during heat treatment are presented in Fig. 1.

α-la immunoreactivity of raw whey was found to be 575±4.71 μg ml^{-1}. In the whey samples heated at 60 or 65 °C, immunoreactivity during the process was lower than that determined in raw whey and ranged from 246±3.91 to 278±1.09 μg ml^{-1} and from 270±1.64 to 452±1.74 μg ml^{-1}, respectively. In the whey samples heated at 70, 72 or 80 °C immunoreactivity varied to a high extent during the process and a marked immunoreactivity increase was observed as compared to raw whey α-la immunoreactivity. The highest immunoreactivity of 1141±5.49 μg ml^{-1} was observed after 10 min of heating at 70 °C, next 2924±10.14 μg ml^{-1} after 5 min of heating at 72 °C and then 3683±11.42 μg ml^{-1} after 5 min of heating at 80 °C. During further heating, the immunoreactivity was reduced. Raising of heating temperature up to 90 °C caused a rapid reduction in immunoreactivity below that of raw whey, and further raise up to 100 °C caused even further reduction.

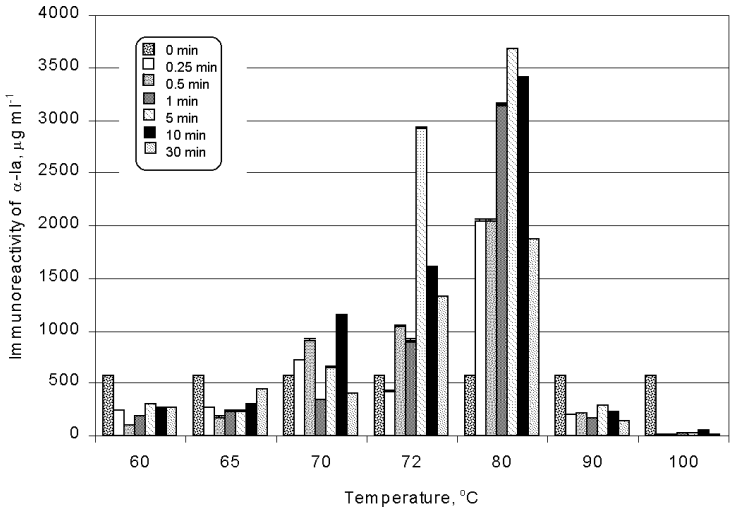

Fig. 1. Changes in the immunoreactivity of α-lactalbumin during heat treatment of whey at 60, 70, 72, 80, 90 and 100 °C

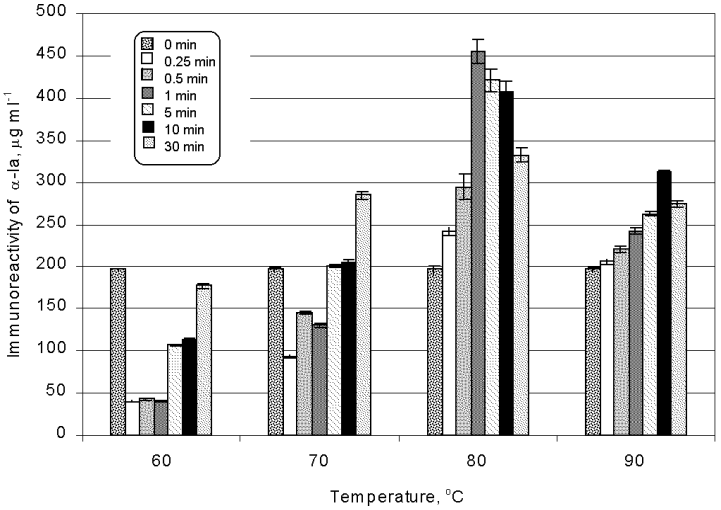

Fig. 2. Changes in the immunoreactivity of α-lactalbumin during heat treatment of a model solution at 60, 70, 80 and 90 °C

As other whey compounds can interact with the protein fraction examined, thereby changing its immunoreactive properties, pure α-la was also heated under the same conditions so that the effects of environmental factors would be excluded. The changes in pure α-la immunoreactivity were similar to that of whey α-la during the heating (Fig. 2). As the heating temperature increased, an increase in immunoreactivity was observed followed by a decrease. It was found that α-la immunoreactivity reached its maximum of 456±15.61 μg ml^{-1} at 80 °C after 1 min of heating.

The changes in α-la content of whey samples during heating were determined by applying ion exchange chromatography in the FPLC system. As a result of chromatographic separation of raw whey proteins, five peaks were obtained (Fig. 3) and three of them were identified. The peak eluting at 38 min corresponded to α-la standard and the other two peaks eluting after 80 min corresponded to β-lactoglobulin standard (β-lg A and β-lg B). The obtained chromatographic pattern of whey proteins is similar to that reported in the literature. LAEZZA and co-workers (1991) obtained a similar pattern of whey protein from Mono Q 5/5 column using linear gradient of elution buffers A: 20 mM TRIS/HCl, pH 7.0, and B: 20 mM TRIS/HCl, pH 7.0 containing 0.35 M NaCl.

In the present work, FPLC analysis was applied to determine the percent changes in α-la content of whey depending on the parameters (i.e., temperature and duration) of the heating process. In raw whey, α-la made up 15%±0.89 of total whey protein (Fig. 4).

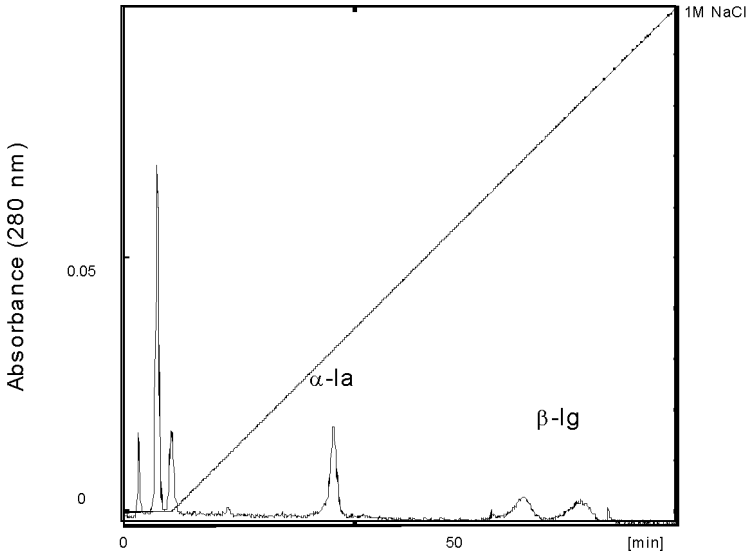

Fig. 3. Ion-exchange chromatography of raw whey protein WP-PEI HR 5/5. Eluting buffers: A: 0.1 M Tris/HCl, pH 7.0 and B: 0.1 M Tris/HCl, pH 7.0 containing 1 M NaCl

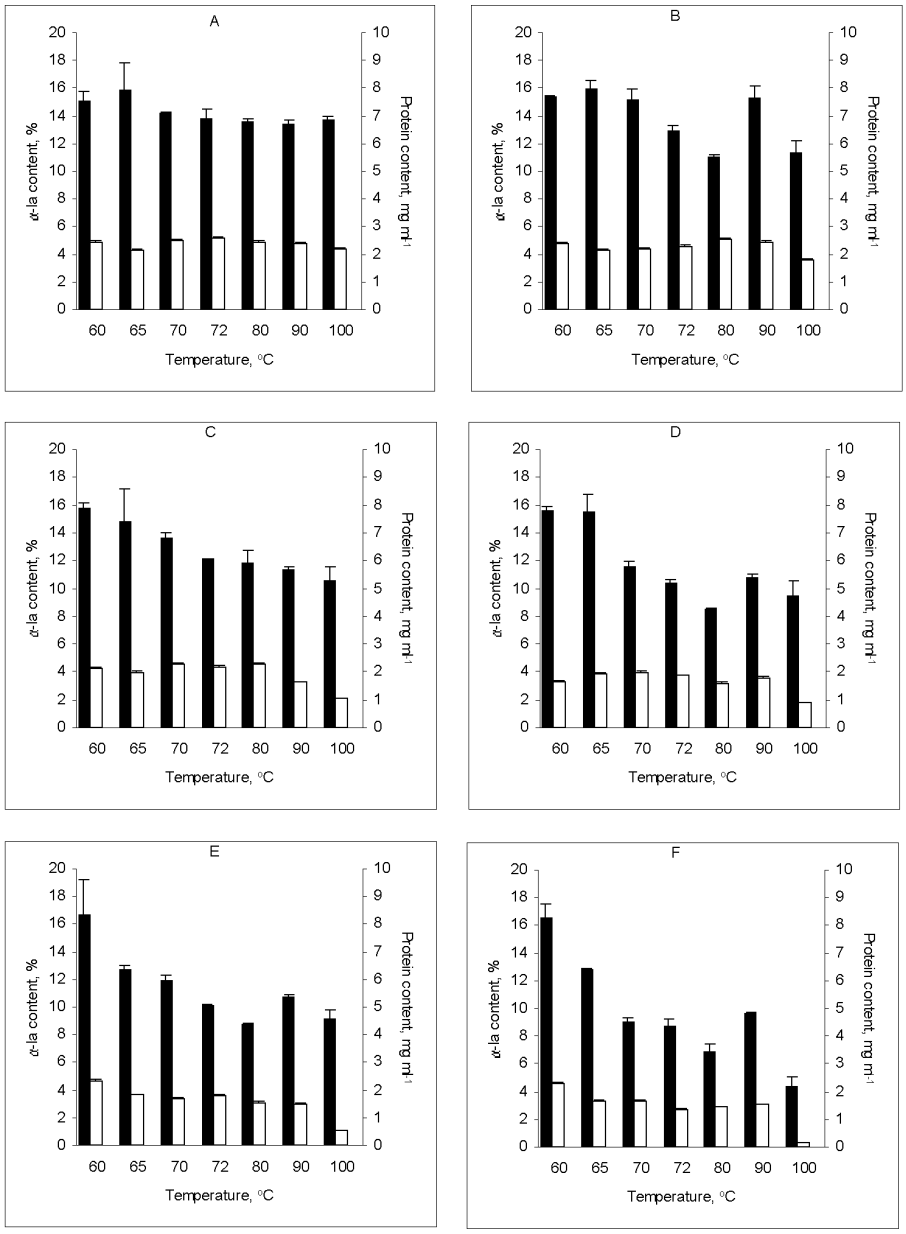

Fig. 4. Total protein content (■) and percentage of α-lactalbumin in whey protein (□) during heat treatment at temperatures of 60, 70, 72, 80, 90 and 100 °C for A: 0.25, B: 0.5, C: 1, D: 5, E: 10 and F: 30 min

The α-la content in whey samples heated at 60 °C ranged insignificantly from 15%±0.63 to 16%±1.03. A slight decrease in its content was observed during heating at 65 °C with time. Significant changes in α-la content were observed during heating at 70 °C. A decrease from 14%±0.1 after 15 s to 9%±0.33 after 30 min of heating was noted. Similar changes from 13%±0.13 after 15 s to 8%±0.49 after 30 min were observed at 72 °C. Heating at 80 °C or 90 °C caused a further decrease in α-la content. The highest decrease in α-la content to 4%±0.69 after 30 min was observed at 100 °C.

It has been demonstrated in our experiment that heat treatment does not always cause a reduction in the immunoreactive properties of proteins and that such factors as temperature and time of heating appear to be essential factors which can affect these properties.

α-la seems to be a quite stable protein during heating at 60 or 65 °C. JU and co-workers (1999) reported that pure α-la was renatured to 80–90% after heating at 60–65 °C and it did not form aggregates. However, approximately twice lower immunoreactivity, as compared to raw whey, may suggest structural changes, which can affect the epitope areas, thus changing immunoreactivity of the protein examined. Renaturation is possible due to the small size of the protein (123 amino acids) and the presence of four disulphide bonds, which restrict the number of conformational states that the protein can assume (JELEN & RATTRAY, 1995). It means that after renaturation α-la can change some of its properties including immunoreactivity. The rate and method of cooling are important factors, which can affect the renaturation process and can be the explanation of this marked discrepancy.

The most intensive changes occurred in the temperature range of 72 to 100 °C, where a high decrease in α-la content was observed with elongation of heating time. A decrease in α-la and total protein contents during heating suggests that heat treatment caused irreversible changes in the protein examined. It can be supposed that in the temperature range of 72–80 °C where the high α-la immunoreactivity was observed and percentage of the protein decreased (Fig. 4), the heat-induced conformational changes along with polipeptide chain denaturation and unfolding revealed additional epitopes, thus affecting immunoreactive properties.

A similar tendency was observed by RAUCH (1996) who heated α-la dissolved in phosphate buffer of pH 7.5 at temperatures of 40, 50, 60, 70, 85 and 90 °C for 2, 5, 10 and 30 min and found the maximum of α-la immunoreactivity at 80 °C, which may have resulted from heat-induced conformational changes in the protein molecule. The four disulphide bonds stabilising the molecule can be reduced, thus affecting the globular tertiary structure. The polipeptide chain is unfolded and epitopes – so far inside the molecule – are exposed and readily recognized by antibody (JEANSON et al., 1999). It is supposed that fragments of the protein molecule other than those containing disulphide bonds are responsible for the immunoreactive properties of α-la.

It is considered that α-la denaturation process at a temperature of 72 °C is irreversible (JELEN & RATTRAY, 1995). According to RELKIN (1996), the irreversible denaturation is a result of hydrolysis reaction of glutamine to glutamic acid and of

disulphide bonds reduction. During heat treatment, thiol-reactive groups are formed, which contribute to the formation of disulphide-linked oligomers. Elongation of heating time increases the average size of oligomers.

Heating at 90 or 100 °C reduced the immunoreactivity of α-la to a high extent.

The ion exchange chromatography showed that α-la contents were highly decreased with elongation of time during heating at these temperatures. These findings are in agreement with our previous results (MIERZEJEWSKA et al., 2002), where capillary electrophoresis was employed to determine the denaturation degree of cow milk α-la during heat treatment of whey. It most likely appears to be caused by heat-induced irreversible denaturation of whey proteins. A decrease in α-la content in acidic whey during heat treatment proves that its solubility is reduced and it can be irreversibly denatured and precipitated (ELFAGM & WHEELOCK, 1978; CORREDIG & DALGLEISH, 1996).

It is supposed that the decrease in immunoreactivity results from the fact that epitopes were denatured or the proteins bearing epitopes formed aggregates and precipitated from solution.

3. Conclusion

In conclusion, there exists the possibility of influencing the immunoreactive properties of α-la by heat treatment. Heating at 100 °C has proved to be highly effective for the reduction of α-la immunoreactivity.

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